SOME EFFECTS OF METHIONINE ON THE UTILIZATION OF NITROGEN IN THE ADULT DOG [1]

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THREE FIGURES

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The relationship between nitrogen balance and absorbed protein nitrogen in dogs and in man is linear in the regions of negative and low positive nitrogen balances (Allison and Anderson, '45; Bricker, Mitchell and Kinsman, '45). The slope of this line is equal to the fraction of food nitrogen retained in the body of the animal and therefore, by definition, to the biological value of the dietary protein provided the excretion of body nitrogen is not affected by the dietary nitrogen. The excretion of body nitrogen, however, was shown to be a variable in protein depleted dogs fed egg white (Allison, Seeley, Brown and Anderson, '46) so that the slope of the line in these experiments was not equal to the biological value of the protein but was some function of it. The feeding of egg white nitrogen to these depleted animals conserved body nitrogen, a finding which is in agreement with data obtained in

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similar experiments on rats (Willman, Swanson, Stewart, Stevenson and Brush, '45). It has been suggested that the slope of the line be called the nitrogen balance index of the dietary protein since the slope is the rate of change of nitrogen balance with respect to nitrogen intake, a function of but not necessarily equal to the biological value (Allison, Seeley, Brown and Anderson, '46; Allison, Anderson and Seeley, '46).

Further information is needed however to evaluate this concept of food nitrogen affecting the excretion of body nitrogen. A review of the literature shows that methionine reduces the excretion of nitrogen in normal adult dogs receiving a protein-free diet (Miller, '44). Stevenson, Swanson, Willman and Brush ('46) have reported also a nitrogen sparing action of methionine in the rat. It seemed possible that this amino acid could effect a nitrogen sparing action in normal dogs similar to that found for egg white in protein depleted animals. Studies were planned therefore, to evaluate the effect of methionine on the excretion of nitrogen and on the nitrogen balance indexes of dietary proteins in normal adult dogs.

METHODS

The isocaloric diets and experimental techniques in these studies were the same as those described previously (Allison and Anderson, '45; Allison, Seeley, Brown and Anderson, '46).

The dogs were healthy normal mongrels, free from parasites. They weighed from 8 to 11 kg. The urine and feces were collected daily, 4-day collections being pooled for analysis. A preliminary feeding period of 4 days preceded the collection period in those experiments in which the nitrogen balance indexes were determined. The urine and feces were analyzed for total nitrogen by the Pregl micro-Kjeldahl method using selenium oxychloride as the catalyst. Urine urea and ammonia were determined by the aeration procedure of Van Slyke and Cullen ('16). The method of Folin ('14) was used to determine the urine creatine and creatinine.

RESULTS

Table 1 is a summary of average data obtained on 3 dogs fed consecutively a protein-free diet, a casein diet, and an egg white diet, dl-methionine being added to each diet during 1 of the 4-day collection periods. The dogs were fed the protein-free diet for 4 days (period 1) during which time the

TABLE 1

Average data obtained on 3 dogs fed dl-methionine and other sources of nitrogen. The data were obtained consecutively, each set representing the average results of a 4-day collection period. Absorbed nitrogen, urinary nitrogen, and nitrogen balance are expressed as gm/day/m² of body surface area.

PERIOD NO.	NITROGEN SOURCE	ABSORBED NITROGEN GM/DAY/M²	URINE NITROGEN GM/DAY/M²	NITROGEN BALANCE GM/DAY/M²
1	protein free	0	2.56	— 3.02
2	dl-methionine [1]	0.24	1.88	— 2.27
3	protein free	0	1.82	— 2.32
4	protein free	0	2.65	— 3.18
5	casein	1.80	2.99	— 1.50
6	casein + dl-methionine [1]	2.04	1.78	— 0.22
7	casein	1.80	1.49	— 0.19
8	casein	1.80	2.33	— 1.04
9	egg white	1.96	1.79	— 0.29
10	egg white + dl-methionine [1]	2.10	1.35	+ 0.35
11	egg white	1.96	1.51	0
12	egg white	1.96	1.52	— 0.04
13	egg white	1.96	1.99	— 0.45

[1] An average of 0.24 gm of dl-methionine nitrogen was fed to each dog each day/m² of body surface. All of the amino acid nitrogen was absorbed.

average excretion of urine nitrogen was 2.56 gm and the nitrogen balance was — 3.02 gm/day/m² of body surface area. An average of 0.24 gm/day/m² of dl-methionine nitrogen was added to the protein-free diet for the next 4 days (period 2). All of the amino acid nitrogen was absorbed. The excretion of urinary nitrogen was reduced during this period to 1.88 gm, the nitrogen balance being increased to — 2.27 gm/day/m². This body nitrogen sparing action of methionine, similar to

that reported by Miller ('44), was carried over into the third period even though the amino acid had been removed from the diet. The excretion of nitrogen returned to control values during period 4.

The protein-free diet was supplemented with casein during period 5. The excretion of nitrogen in the urine increased during this period to 2.99 gm/day/m², an increase which would be expected from previous studies on the nitrogen balance index of casein (Allison and Anderson, '45). Addition of the same amount of methionine to this casein diet (period 6) as was previously added to the protein-free diet resulted in a marked decrease in the excretion of urinary nitrogen (to 1.78 gm/day/m²) and an increase in the nitrogen balance almost to equilibrium (to — 0.22 gm/day/m²). Supplementation with methionine reduced the excretion of nitrogen more when the dogs were fed the casein than when they were fed the protein-free diet. These data can be interpreted to mean that methionine is supplementing both body and casein nitrogen. The reduction in excretion of nitrogen was carried over into period 7 when the dogs received casein unsupplemented with methionine.

The excretion of urinary nitrogen and the nitrogen balance in period 8 were approaching the initial values for casein (period 5) when egg white was substituted for casein in period 9. Egg white caused a decrease in the excretion of urinary nitrogen (period 9) from that previously found for casein (period 8). This decrease in excretion of urinary nitrogen through feeding egg white agrees with previous experiments (Allison, Seeley, Brown and Anderson, '46). The addition of methionine to egg white (period 10) caused urinary nitrogen to decrease and the nitrogen balance to increase but these changes were not as marked as in the experiments on the casein diet. As in the previous experiments the sparing action was carried over into periods when the diet contained no additional methionine above that present in the egg white.

Figure 1 illustrates average urinary nitrogen excretions in 3 dogs receiving the protein-free diet for 4 days and then the

protein-free diet supplemented with methionine for 3 4-day periods (blocks with slanted lines) followed by the unsupplemented diet again. Approximately 0.24 gm of methionine nitrogen per day per square meter of body surface area was added to the protein-free diet. These data demonstrate that the urinary nitrogen excretion remains depressed when the feeding of methionine is extended from 4 days used in the experiments recorded in table 1 to 12 days. Again the nitrogen excretion remained below control levels for several days after the methionine was removed from the diet.

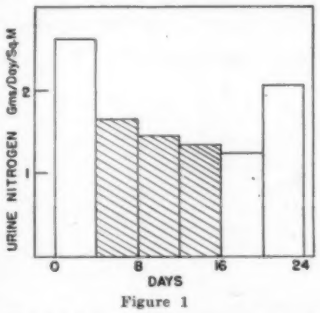

Figure 1

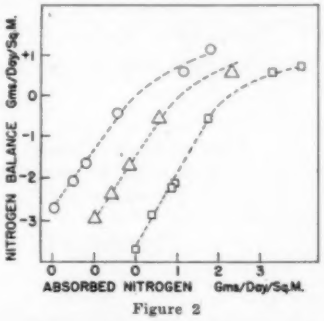

Figure 2

Fig. 1 Average daily excretion of urinary nitrogen in 3 dogs fed protein-free diet (white blocks) and protein-free diet plus 0.24 gm of dl-methionine nitrogen/day/m² body surface area (diagonal lines).

Fig. 2 Absorbed nitrogen (gm/day/m²) vs. nitrogen balance (gm/day/m²). These data were obtained while feeding 3 dogs casein diet supplemented with methionine in the ratio of 1 gm of methionine nitrogen to 14 gm of casein nitrogen.

The effect of the nitrogen sparing action of methionine on the nitrogen balance index is illustrated in figure 2 by average data obtained on 3 dogs fed casein supplemented with dl-methionine (1 gm of dl-methionine nitrogen to 14 gm of casein nitrogen). The relationship between nitrogen balance and absorbed nitrogen is linear in the region of negative nitrogen balance, becoming curvilinear in the region of positive nitrogen balance. The curvilinearity originates closer to zero nitrogen balance in methionine-supplemented than unsupplemented casein. The average slope of these lines in the

region of negative nitrogen balance is 1.5 almost double the nitrogen balance index of 0.8 for unsupplemented casein.

The nitrogen sparing action of methionine can be illustrated by applying this nitrogen balance index to the following equation derived previously (Allison, Seeley, Brown and Anderson, '46).

$$EN = NE_o - AN\ (K\text{–}BV) \qquad (1)$$

where EN is the nitrogen excreted from body sources, NE_o the excretion of nitrogen on a protein-free diet, AN the absorbed nitrogen, K the nitrogen balance index and BV the fraction of nitrogen retained in the body of the animal. Since BV can never be greater than unity the value for K of 1.5 results in a positive figure in the parenthesis of equation (1). Thus the

TABLE 2

Average nitrogen balance indexes determined on groups of 3 dogs fed the casein diet alone and the casein diet supplemented with various amounts of methionine.

METHIONINE N / CASEIN N	NITROGEN BALANCE INDEX
0.000	0.78
0.004	0.86
0.008	0.95
0.025	1.58
0.071	1.50

excretion of body nitrogen EN decreases as absorbed nitrogen (AN) increases. Feeding casein supplemented with methionine spares body nitrogen in the normal dog, therefore, even as egg white spares body nitrogen in the depleted animal. The curvilinearity of the lines in the region of positive nitrogen balance (fig. 2) proves that the sparing action decreases rapidly as nitrogen intake increases in this region. The maximum sparing of nitrogen by methionine supplemented casein is, therefore, in the region of nitrogen equilibrium.

The data in table 2 demonstrate the effect of adding different amounts of dl-methionine on the nitrogen balance index

of casein. As the ratio between methionine and casein nitrogen increases the nitrogen balance index increases gradually from approximately 0.8 for unsupplemented casein to a maximum around 1.5 for supplemented casein.

The data in figure 3 illustrate the relationship between urinary nitrogen excretion and methionine nitrogen intake, when the amino acid was added to the diet containing a constant amount of casein nitrogen (3 gm/day/m² of body surface area). These data demonstrate that as the amount of methionine nitrogen added to the casein increases the excretion of urinary nitrogen decreases rapidly to a minimum and constant value. This minimum value of excretion coincides

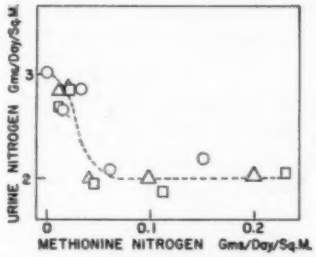

Fig. 3 Urinary nitrogen excretion (gm/day/m²) in 3 dogs fed a diet supplemented with different amounts of methionine nitrogen but containing a constant amount of casein nitrogen (3 gm/day/m² of body surface area).

with the establishment of the constant maximum nitrogen balance index of 1.5 to 1.6 recorded in table 2. There is then a definite and fixed amount of methionine necessary to produce the maximum sparing action of the casein body nitrogen mixture. Emphasis is put upon this mixture because no real separation can be made between the dietary and body nitrogen. Schoenheimer and Rittenberg ('40), for example, have demonstrated a dynamic equilibrium between tissues and the surrounding media which makes the separation of food nitrogen from body nitrogen a difficult task. The methionine is supplementing body nitrogen alone when fed with protein-free diet, but it supplements a new pattern made up of dietary

as well as body nitrogen when fed with casein or with egg white.

The data summarized in table 3 show that the decrease in the excretion of urinary nitrogen which accompanies the addition of methionine to casein is due to a decrease in the excretion of urea and not of ammonia, creatinine, or creatine. Thus methionine affects the metabolic paths involved in the synthesis of urea altering the ratio between excretion of ammonia and urea.

TABLE 3

Average data obtained on 3 dogs fed approximately 3 gm of casein nitrogen per day per square meter of body surface area. Different amounts of dl-methionine (recorded in column 1) were added to the casein. Each set of data represents the average excretion over a period of 4 days. The data were obtained consecutively with a 4-day adjustment period when the diet was changed in methionine concentration.

DL METHIONINE MG/DAY	URINARY NITROGEN			
	Creatinine mg/day/m²	Creatine mg/day/m²	Ammonia mg/day/m²	Urea mg/day/m²
0	167	35	372	1974
0	169	37	401	2054
50	157	42	407	1612
100	185	31	402	1516
0	169	36	404	1700
200	172	42	361	891
500	170	31	410	805
0	162	40	423	1646
1000	155	48	530	796

SUMMARY

1. The addition of dl-methionine to a protein-free diet or to diets containing casein or egg white will reduce the excretion of nitrogen in adult dogs. The excretion remains lower than control values for several days after the amino acid has been removed from the diet. The addition, therefore, of methionine to these diets spares nitrogen in the animal, the sparing action being more marked in the presence of casein than of egg white.

2. The relationship in normal adult dogs between nitrogen balance and absorbed casein nitrogen supplemented with

methionine is linear in the region of negative nitrogen balance and curvilinear in the region of positive nitrogen balance.

3. The slope of the line in the region of negative nitrogen balance (nitrogen balance index) increases from 0.8 in unsupplemented to from 1.5 to 1.6 in methionine supplemented casein. Slopes greater than unity prove that the addition of methionine to casein spares body and dietary nitrogen in the animal. This sparing action decreases rapidly in the region of positive nitrogen balance where the slope is a decreasing variable.

4. A definite quantity of methionine is required to produce the maximum nitrogen sparing action. The addition of 0.025 gm of methionine nitrogen to 1.0 gm of casein nitrogen produces a maximum nitrogen balance index of approximately 1.5. The addition of larger quantities of methionine to the casein does not alter this index.

5. Methionine produces the nitrogen sparing action through a reduction in the excretion of urea nitrogen, the ratio between ammonia nitrogen and urea nitrogen being increased.

ACKNOWLEDGMENT

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THE EFFECT OF METHIONINE UPON THE URINARY NITROGEN IN MEN AT NORMAL AND LOW LEVELS OF PROTEIN INTAKE [1]

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Studies of the nitrogen excretion in rats and dogs on low levels of dietary nitrogen have shown that the addition of egg white protein to the diet results in a reduction of the nitrogen excretion which is not produced by casein (Swanson, '46; Allison, '45). It had previously been shown that a part of the nitrogen of certain amino acids fed singly to dogs on a low-protein diet is retained or spares body protein (Nielsen et al., '39). The same situation was found to obtain in rats (Swanson, '46), and the nitrogen-sparing effect of methionine

[1] This work was done under a contract between the University of Southern California and the Research and Development Branch, Military Planning Division of the Office of the Quartermaster General, U.S.A.

The subject matter of this paper has been undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

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[3] We wish to thank Mr. W. R. Harriman, Director, and Dr. Douglas W. Ritchie, Medical Director, for making the arrangements essential to this study; and to acknowledge the invaluable assistance of Miss Edmonda Hughes, Head Dietitian, in the planning and preparing of the diets.

in dogs has been confirmed (Allison, '45, '46). In these later animal experiments, the amount of body protein nitrogen which is spared is greater than the methionine nitrogen ingested. Such an effect would be of considerable importance in human nutrition under conditions of restricted food and water intake. Body protein would be conserved and less water would be required for the excretion of urea and other solutes as the result of including a relatively small amount of a single amino acid in the restricted diet.

The present studies were undertaken to determine whether such an effect is produced by methionine in men. Since it is to be expected that such an effect will most probably be observed when the caloric needs are met from non-protein sources, the bulk of the experiments were carried out at relatively high caloric intakes with protein intake of 75 gm (Diet A) or restricted to 14 gm per day (Diet B). A few experiments were also carried out at a lower caloric level, with the protein intake reduced to about 5 gm per day (Diet C). It should be recognized at the onset that the experimental periods were not prolonged, and that the subjects began each restricted period without depletion of protein stores. The conclusions can only be applied to short periods of protein restriction, and may be modified if it is possible to study subjects whose protein stores have been depleted. However, the conditions are comparable to those under which an effect has been noted in experimental animals.

EXPERIMENTAL

Subjects

The major part of the study was carried out upon 8 male volunteers who were patients at the Rancho Los Amigos. These men ranged in age from 27 to 53 years, and were not suffering from ailments which would be expected to alter their nitrogen metabolism. This expectation was borne out by the constancy of the metabolic data obtained, except for the case of subject no. 13, who showed definite evidences of metabolic disturbances during the latter part of the experiments.

The supplementary experiments at more restricted protein intakes were carried out on 4 healthy, adult, male research workers in the same age group as the other subjects.[4]

Control of the subjects. The subjects at the Rancho Los Amigos were confined to 1 ward which was used exclusively for this experiment. A nurse was in attendance at all times, and during the day 2 nurses and an attendant were on duty. Subjects were allowed to leave the ward for exercise, but only under supervision.

The normal activities of the second group of subjects were not modified during the experimental periods.

Diets

The diets were prepared in the diet kitchen of the Rancho Los Amigos, and portions were weighed onto the individual trays at the ward. The entire meal was eaten by the subjects.

Three daily menus were selected for the basal (adequate protein) series (Diet A) and 2 daily menus for the experimental (low protein) periods (Diet B). Both menus were calculated to provide 2000 Cal. The adequate protein diets were calculated to supply 12 gm of protein nitrogen per day, and were found to contain 11.1 to 12.6 gm on analysis. The low-protein diets were designed to provide 2 gm of nitrogen per day, and actually contained 2.2 to 2.3 gm nitrogen. It was not considered that an adequately palatable diet could be provided with casein as the sole source of protein, but 65% of the protein was supplied by whole milk proteins. The 2 menus for the low-protein periods are given in table 1.

In the supplementary experiments, at very low levels of protein intake, the diet consisted of 1 liter of grapefruit juice per day, containing 0.94 gm of nitrogen and supplying 400 Cal., with sucrose to supply an additional 200 Cal.; or additional sucrose and glucose candies to supply 800 Cal. (Diet C).

[4] The authors wish to thank Dr. Richard J. Winzler and Mr. Fred H. Mattson who served as subjects.

TABLE 1

Composition of the low protein diets (Diet B).

MEAL	DIET 1		DIET 2	
Breakfast	Grapefruit juice	180 gm	Orange juice	185 gm
	Cornflakes	15 gm	Rice Krispies	15 gm
	Sucrose	14 gm	Karo syrup	50 gm
	Cream, 21%	160 gm	Cream, 21%	160 gm
	Coffee	1 cup	Coffee	1 cup
	Glucose candy	11 gm	Glucose candy	16 gm
Dinner	Salad		Salad	
	Canned pear	81 gm	Lettuce leaf	38 gm
	Lettuce leaf	38 gm	Salad dressing	10 gm
	Salad dressing	10 gm	Fresh grapefruit	53 gm
	Pudding		Strained honey	15 gm
	Dry rice	20 gm	Margarine	13 gm
	Raisins	29 gm	Biscuit [1]	70 gm
	Sucrose	14 gm		
			Lemonade	
	Margarine	13 gm	Lemon juice	60 gm
	Biscuit [1]	70 gm	Sucrose	10 gm
	Coffee	1 cup		
Supper	Salad		Salad	
	Lettuce leaf	38 gm	Lettuce leaf	38 gm
	Salad dressing	10 gm	Salad dressing	10 gm
	Cobbler		Sweet potato	81 gm
	Brown sugar	27 gm	Margarine	13 gm
	Apple, peeled	100 gm		
	Biscuit [1]	70 gm	Pudding	
	Margarine	13 gm	Brown sugar	27 gm
			Tapioca	15 gm
	Whole milk	100 gm	Pineapple	94 gm
	Glucose candy	20 gm		
			Whole milk	100 gm
			Glucose candy	37 gm

[1] Biscuit recipe furnished through the courtesy of Dr. Pearl Swanson.

Corn starch	50 gm
Crisco	16 gm
Buttermilk	30 gm
Soda	¼ tsp.

Analytical methods

Nitrogen determinations were made by the Kjeldahl method. The inorganic and ethereal sulfate were determined gravimetrically, as barium sulfate, and the methionine was determined by the method of Albanese et al. ('44). Hemoglobin and plasma proteins were determined by the copper sulfate specific gravity method (Phillips et al., '43). Hemoglobin was also determined colorimetrically as acid hematin and plasma proteins by the biuret method (Mehl, '45).

Plan of the experiments

The subjects to be studied at the nitrogen intake of 2 gm were first placed on the adequate-protein diet for a period of 2 weeks. During this period and subsequently, daily 24-hour urine samples were collected, and stools were weighed but not kept for analysis. Daily records were kept of body temperature, pulse rate, respiration rate, and physical activity. The weight, blood pressure, blood count, hemoglobin, and plasma protein were obtained at weekly intervals. The nitrogen content of the diets was determined by weighing out 1 extra tray, homogenizing the food from 3 meals in a Waring blendor, and taking an aliquot for the analysis.

The preliminary period was then extended for 10 days, during which 3 gm of dl-methionine were added to the diet daily. This served to provide a somewhat longer period on Diet A, and at the same time provided information regarding the effect of methionine in subjects in nitrogen equilibrium. The methionine was then discontinued for 10 days, after which the low-protein period (Diet B) was begun. Four of the subjects were given a daily supplement of 3 gm of dl-methionine during this first low-protein period and the other 4 subjects received the low-protein diet alone. The experimental period of 10 days was followed by a recovery period of 10 days during which the subjects were returned to the adequate-protein intake, and methionine discontinued. A second 10-day experimental period was then instituted. This

provided a control period for the 4 subjects who had previously received the methionine supplement, and a methionine supplement period for the other 4 subjects. The entire experiment was concluded by a final 10-day recovery period.

The very low-protein diets (Diet C) were undertaken in a less comprehensive way, in the hope that some effect of methionine could be demonstrated at these very low levels of nitrogen intake. Laboratory workers went on the low-protein diet following their customary dietary regime, and carried on with their usual duties. One subject, no. 14, carried through 2 experimental periods of 10 days each, separated by a recovery period of 3 weeks. During the first low-protein period, a daily supplement of 3 gm of dl-methionine was provided, while the second period served as a control. Subject no. 15 received the methionine supplement during the first 7 days of a 10-day low protein period, and subject no. 16 received the methionine during the last 3 days of a 10-day period. Subject no. 17 was provided with the daily supplement of 3 gm of dl-methionine for the entire 10-day period. The data obtained on these last 4 subjects were limited to the 24-hour urinary nitrogen, inorganic and ethereal sulfate, and methionine excretions, and the body weights.

The choice of a 3-gm daily supplement of methionine is somewhat arbitrary, but was determined primarily by the levels of the methionine supplement required to give a maximum effect in rats and dogs. This amount is somewhat higher than that of 1.9 gm per day, suggested by Stare, Hegsted and McKibben ('45) as being required by a 70-kg man, though it is not sufficient to meet the requirement of approximately 4 gm postulated by Block ('43).

RESULTS

The results of the experiments employing the 2000-Cal. diet and 2 gm of nitrogen during the low-protein periods will be discussed first, since they are the most nearly complete. The serum protein, hemoglobin, and body weight have not been tabulated, since they showed no significant change during the

course of the experiments. The body weight, for example, did not vary by more than 4% during the entire period. The greatest interest attaches to the data on nitrogen and sulfur excretion. The results obtained on the basal diets are shown in table 2. Average values for the entire group are reported, and only averages for 5-day periods are recorded. Urine methionine was not determined during this period, but the urinary nitrogen, sum of ethereal and inorganic sulfate,

TABLE 2

Summary table of the effect of methionine at an adequate protein intake (Diet A).

CHEMICAL ANALYSES	PERIOD I BASAL DIET			PERIOD II BASAL DIET PLUS 3 GM METHIONINE DAILY			PERIOD III BASAL DIET	CHANGE
	Mar. 19–Mar. 23	Mar. 24–Mar. 28	Average	Mar. 29–Apr. 2	Apr. 3–Apr. 8	Average	Apr. 12–Apr. 15 [1]	Average of II minus I plus III
Urine N, gm/24 hr.	9.94	9.59	9.76	10.34	10.23	10.29	9.47	+ 0.63
Urine SO_3 [2], gm/24 hr.	1.48	1.45	1.47	2.68	2.77	2.72	1.49	+ 1.24
Urine creatinine, gm/24 hr.	1.11	1.01	1.06	0.97	0.99	0.99	1.03	— 0.07
Food N, gm/24 hr.	12.29	11.42	11.77	12.55	11.20	11.88	11.13	+ 0.46

[1] No collections were made from April 9 to April 11, inclusive, although the subjects remained on the basal diet.

[2] Sum of inorganic and ethereal sulfate.

creatinine and food nitrogen are tabulated. The change produced by methionine is indicated in the last column of table 2, and is calculated from the difference between the averages of basal periods I and III and the basal period plus methionine, period II. There was a small increase in urinary nitrogen excretion, 0.63 gm per day, which was largely accounted for by the difference in the food nitrogen, 0.46 gm. The excess excretion of 1.24 gm SO_3 accounts for 77% of the extra sulfur ingested in the methionine, and, making allowances for the

probable excretion of sulfur in other forms, it corresponds to essentially complete metabolism of the ingested methionine.

The data obtained on the low-protein diet are summarized in table 3. Here again, averages are given for 5-day periods, except for the first basal period preceding the low-protein diet, which includes values for only 4 days. The average decrease in urinary nitrogen excretion in changing from a diet providing 11.9 gm of nitrogen to one containing 2.2 gm was 5.0 gm. The inclusion of 3 gm of dl-methionine in the low-protein period resulted in a smaller decrease in urinary nitrogen, averaging 4.7 gm per day. The smaller decrease with methionine can be accounted for by the 0.3 gm of nitrogen contained in the methionine. Likewise, the extra sulfur excretion corresponds to 82% of that ingested in the methionine, and making allowances for the fact that some additional neutral sulfur was not accounted for, this corresponds to essentially complete excretion of the methionine sulfur.

The nitrogen excretions have been recalculated in terms of the grams of nitrogen per square meter per 24 hours, and are included in table 4. These values serve to indicate the relative constancy of the results obtained with individual subjects (except for the obvious abnormalities in the case of subject no. 13), and provide some basis for comparison with the data obtained on dogs. Again, the nitrogen excretions are slightly greater with the methionine supplement. Excluding subject no. 13, the average difference was 6.1% during the first 5 days ,and 3.6% during the second 5 days on the low protein diet.

The results of the experiment with subject no. 14 are given in table 5. The two 10-day experimental periods employed a 600 Cal. diet, with a nitrogen intake of 0.94 per day. Averages of the nitrogen excretion during the last 5 days were 5.57 gm per day without methionine, and 7.12 gm per day with 3 gm of methionine daily. A total of 6.5 gm of methionine sulfur were ingested during these 5 days, and 6.3 gm more sulfur were excreted than in the control period.

TABLE 3

Summary table of the effect of methionine during a protein-low diet (Diet B).

CATEGORY OF INTEREST	METHIONINE EXPERIMENT					CONTROL EXPERIMENT					CHANGE IN URINE EXCRETION [2]	
	Basal I [1]	Low-protein-period			Basal II [1]	Basal III [1]	Low-protein-period			Basal IV [1]	Methionine experiment	Control experiment
		1–5	6–10	Average 1–10			1–5	6–10	Average 1–10			
Body wt., lb.	137	....	....	136	137	137	....	....	136	136	−1	−0.5
Food nitrogen, gm	11.9 ± 0.3	....	....	2.23 ± 0.04	11.9 ± 0.3	11.9 ± 0.3	....	....	2.23 ± 0.04	11.9 ± 0.3		
Urine vol., ml	1738	1398	1373	1386	1765	1776	1349	1448	1398	1793	−366	−386
Urine nitrogen, gm	9.30	4.30	3.20	3.75	7.61	10.01	4.60	3.22	3.91	7.98	−4.71	−5.04
Urine sulfate,[3] gm SO_3	1.49	1.88	1.78	1.83	1.38	1.49	0.64	0.58	0.61	1.28	+0.39	−0.77
Urine methionine, gm	0.28	0.65	0.60	0.63	0.34	0.36	0.36	0.31	0.34	0.33	+0.32	−0.01
Urine creatinine	1.04	0.98	1.02	1.00	1.13	1.05	0.98	1.07	1.02	1.15	−0.08	−0.08

[1] Periods immediately preceding or following the low-protein diet (Diet B). All are 5 days except Basal I which was 4 days. Basal periods are those where Diet A was employed.

[2] Average of the experimental periods minus the average of the preceding and following basal period.

[3] Sum of inorganic and ethereal sulfate.

TABLE 4

Summary table of individual levels of nitrogen excretion per unit of body surface expressed as grams of nitrogen per square meter per 24 hours.

SUBJECT NUMBER	SURFACE AREA	BASAL	LOW PROTEIN		BASAL		LOW PROTEIN		BASAL	
		April 12–15	April 16–20	April 21–25	April 26–May 1	May 2–6	May 7–11	May 12–16	May 17–21	May 22–23
	m^2									
1	1.86	5.08	2.90 [1]	1.88 [1]	4.53	6.00	2.68	1.88	4.77	5.57
5	1.68	5.78	3.01 [1]	2.02 [1]	4.62	5.70	2.67	1.85	5.09	4.96
7	1.51	6.37	3.11 [1]	2.34 [1]	5.43	6.17	2.75	1.99	5.38	6.25
9	1.70	5.92	3.00 [1]	1.98 [1]	4.57	5.84	2.97	1.90	4.53	5.87
6	1.71	5.83	2.80	1.71	4.63	5.07	2.83 [1]	1.74 [1]	4.44	5.76
8	1.85	5.70	2.35	1.76	4.20	5.65	2.65 [1]	1.88 [1]	4.08	5.77
10	1.70	5.97	2.82	2.15	4.95	5.78	2.74 [1]	1.91 [1]	5.48	6.33
13	1.74	3.69	2.39	1.77	3.31	3.83	2.14 [1]	1.18 [1]	2.45	3.25

[1] Methionine periods.

The results of the other experiments employing this very low-protein intake are also recorded in table 6, where the urinary nitrogen excretions are again calculated per square meter of body surface. Urinary sulfates, as SO_3, and methionine excretions per 24 hours are also reported for subjects 15, 16, and 17. The excretion of urinary nitrogen does not reach the low values attained on the 2000-Cal. diets, but appears to respond in the same way to the addition of methionine to the diet, with a small increase.

TABLE 5

Effect of methionine on a "protein-free" diet (Diet C). Data obtained from subject no. 14 whose diet furnished 0.94 gm N and 600 Cal. per day.

DAYS ON DIET	BODY WT.		URINE N		CREATININE		SULFATE AS SO_3 [1]		METHIONINE	
	M [2]	C [3]	M	C	M	C	M	C	M	C
	lbs.	*lbs.*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
1	202	198	8.72	11.47	0.73	0.74	2.64	1.41	0.96	0.47
2	201	196	7.98	9.07	0.70	0.65	2.66	1.08	0.83	0.58
3	199	196	9.68	8.41	0.73	0.70	2.54	1.08	0.94	0.62
4	198	195	9.23	7.82	0.70	0.74	2.57	0.92	0.95	0.62
5	197	194	10.29	8.32	0.70	0.79	2.54	0.97	1.14	0.64
6	195	192	7.86	6.22	0.68	0.76	2.31	0.86	0.75	0.55
7	195	191	6.25	4.87	0.65	0.78	2.43	0.85	0.80	0.56
8	194	190	6.93	5.71	0.61	0.66	2.39	0.87	0.78	0.55
9	193	189	7.66	5.65	0.69	0.68	2.24	0 85	0.80	0.61
10	192	188	6.89	5.41	0.70	0.65	2.16	0.79	0.83	0.57

[1] Sum of ethereal and inorganic sulfate.
[2] Methionine (3 gm).
[3] Control.

DISCUSSION

The results of the experiments carried out on the 2000-Cal. diet are sufficiently consistent to conclude that the result of adding 3 gm of methionine per day had the same effect whether the nitrogen intake was 12 gm per day (Diet A), and the subjects in nitrogen balance, or whether the nitrogen intake was 2 gm per day (Diet B), and the subjects in negative nitrogen balance. In either case, the sulfur excretion increases

TABLE 6

Effect of methionine on a "protein-free" diet (Diet C).

DAYS ON DIET	URINE N (GM/M²/24 HR.)						URINE SO_4 (GM SO_3/24 HR.)			URINE METHIONINE GM/24 HR.			BODY WEIGHT (LBS.)		
	No. 14 600 Cal.	No. 14 600 Cal.	No. 14 [1] 2000 Cal.	No. 15 600 Cal.	No. 16 1200 Cal.	No. 17 1200 Cal.	No. 15 600 Cal.	No. 16 1200 Cal.	No. 17 1200 Cal.	No. 15 600 Cal.	No. 16 1200 Cal.	No. 17 1200 Cal.	No. 15	No. 16	No. 17
1	4.15 [2]	5.52	4.91	5.13 [2]	5.97	5.21 [2]	1.31 [2]	1.30	2.08 [2]	0.37 [2]	0.45	0.68 [2]	150	161	169
2	3.82 [2]	4.38	4.13	4.87 [2]	4.81	4.87 [2]	1.73 [2]	1.05	2.40 [2]	0.56 [2]	0.36	0.79 [2]	148	159	167
3	4.65 [2]	4.06	3.71	4.08 [2]	3.72	4.86 [2]	1.95 [2]	0.99	2.27 [2]	0.74 [2]	0.42	0.82 [2]	147	157	165
4	4.43 [2]	3.78	3.05	3.94 [2]	4.04	3.77 [2]	2.14 [2]	1.01	2.12 [2]	0.90 [2]	0.52	0.80 [2]	146	157	164
5	4.94 [2]	4.03	2.77	3.48 [2]	3.53	3.93 [2]	1.92 [2]	0.94	2.22 [2]	0.85 [2]	0.53	0.81 [2]	145	156	164
6	3.79 [2]	3.03	2.44	3.56 [2]	3.12	3.47 [2]	2.57 [2]	0.85	1.80 [2]	0.90 [2]	0.61	0.67 [2]	144	155	...
7	3.02 [2]	2.73	2.23	...	...	3.13 [2]	...	...	2.31 [2]	...	...	0.73 [2]	143	...	163
8	3.36 [2]	2.80	1.98	3.58 [2]	2.26 [2]	2.57	1.67 [2]	0.61 [2]	0.73	1.02 [2]	0.60 [2]	0.50	142	154	162
9	3.72 [2]	2.76	1.57	2.64 [2]	2.71 [2]	2.62	1.12 [2]	1.87 [2]	0.73	0.56 [2]	0.74 [2]	0.51	142	153	161
10	3.37 [2]	2.67	1.66	2.93 [2]	2.73 [2]	2.05	1.50 [2]	2.03 [2]	0.54	0.73 [2]	0.91 [2]	0.33	141	152	160
Average last 3 days	3.49 [2]	2.74	1.74	3.05 [2]	2.57 [2]	2.41									

[1] Data obtained 19 years earlier on the same subject (Deuel et al., '28).

[2] Days on which methionine was taken.

by an amount within 10% of the amount of methionine sulfur ingested and the nitrogen excretion increases slightly. The extra nitrogen, particularly in the low-protein period, can be stated to represent essentially all of the ingested methionine nitrogen. There is, thus, no indication that the added methionine spares body protein, or that its sulfur is utilized. The experiments on very low nitrogen intakes bear out this conclusion completely, and suggest a species difference from the rat and the dog.

The different results obtained with the human subject require some explanation, but must depend primarily upon an explanation of the ability of methionine or any other single amino acid to reduce the urinary nitrogen excretion on an inadequate protein intake. There are 2 similar explanations for such an effect, both of which would postulate a requirement for the amino acid in question which is not met by the breakdown of body protein and the diet. This could be a requirement for some specific function other than protein re-synthesis, or it could represent a requirement for re-synthesis of more important body proteins at the expense of less important ones. In an earlier experiment on a protein-free diet, supplying 2000 Cal., a urinary nitrogen excretion of 0.94 gm/m²/24 hr. was reached (Deuel et al., '28). This is significantly lower than any level of nitrogen excretion reached in Allison's ('46) experiments with dogs. In either case, extra body protein would be broken down to meet this requirement, and a minimal breakdown could only be obtained when these special requirements were met by an extra dietary supplement. In the case of the human experiments, then, it would be concluded that the methionine requirement is lower, and is not a limiting factor in the attainment of minimum nitrogen excretions in these experiments, or that the requirement is met by the body protein breakdown plus any dietary protein. In this connection, it is interesting to compare the levels of urinary excretion of 1.5 to 1.9 gm N/m²/24 hr. reached in a comparable time with dogs receiving a methionine supplement with the average levels of approximately 1.9 gm N/m²/24 hr.

reached by these human subjects without methionine during the last 5 days of the low-protein period.

Since the addition of further methionine did not reduce the nitrogen excretion on the low-protein diet (Diet B), it can be concluded that no more methionine is required under these circumstances than that represented by the entire sulfur excretion, or 1.4 gm methionine per day for our average subject. On the average, the methionine nitrogen (calculated from the total sulfur excretion) per gm of total urinary nitrogen during the last 5 days of the low protein period at 2000 Cal. is 0.041, and this ratio is 0.035 for the last 2 days of the experiments at 600 and 1200 Cal. with subjects no. 14 and no. 17, and also on the sixth and seventh day of the low-protein experiment with subject no. 16. Since the addition of methionine did not further reduce the urinary nitrogen, this ratio is indicative of the maximum methionine requirement at these levels of nitrogen intake. The ratio obtained in a similar way for the basal periods averages about 0.035. The minimum excretion of urinary nitrogen (2 gm/m^2/24 hr.) in dogs (Allison, '46) on an intake of 3 gm of casein N per m^2 per day was attained with a supplement of 0.04 gm of dl-methionine N per m^2. Since the casein furnished an additional 0.57 gm of methionine (Dunn et al., '46) or 0.053 gm of methionine-N per m^2 per day, the total methionine-N was at least 0.1 gm for a urinary N of 2 gm, or 0.05 gm of methionine-N per gm of urinary nitrogen. If allowance is made for the fact that the dogs were in positive nitrogen balance, the sulfur stored in protein must also be considered, and this would reduce the estimate of the ratio of methionine nitrogen to urinary nitrogen to about 0.033. Although the estimate of the ratio of methionine-N to the total urinary-N may probably be too high in the case of the men and though that for the dogs may be somewhat too low, it can be argued that these calculations indicate an appreciably higher methionine requirement in dogs than in man unless some further assumption is made regarding the fraction of the urinary sulfur which is really derived from methionine in the human experiments. It may be of some interest to note

that the ratio of urinary S to N increased during the course of the present protein-low or protein-free experiments. This increase is even more marked in the latter periods of the experiment of Deuel et al. ('28) in which the neutral sulfur excretion was relatively constant, and averaged about 0.1 gm/S/24 hr. If all of this were methionine, it would correspond to 0.46 gm/24 hr., and compares favorably with the methionine excretion of 0.6 gm obtained with the same subject in the present experiments (table 5). This excretion appears to be quite constant at different levels of protein intake (tables 3, 5, and 6), and therefore appears to be independent of the methionine intake, except insofar as the ingestion of large amounts of the amino acid itself leads to some spilling over into the urine. The ratio of sulfate-S to urinary nitrogen remains quite constant in the present experiments, but increases significantly in the longer experiment of Deuel et al. ('28) During protein deprivation, then, the body protein which is broken down becomes increasingly richer in sulfur-containing amino acids. However, the present experiments suggest that the human body is not limited in its ability to conserve nitrogen by the need to meet a methionine requirement.

The comparison of the present experiments with a 2000-Cal. intake and the 600- and 1200-Cal. intakes, as well as with the earlier experiment of Deuel et al. ('28), demonstrates the importance of an adequate calorie intake in the attainment of a minimal nitrogen excretion on a low-protein diet. The calorie intake required to establish a minimum nitrogen excretion cannot be fixed definitely from these experiments, but it can be concluded that it is greater than 1200 Cal.

SUMMARY AND CONCLUSIONS

1. Experiments have been carried out on human subjects during 10-day periods, to test the effects of added methionine on urinary excretion. Diets supplying 2000 Cal. and 75 gm of protein (Diet A), 2000 Cal. and 14 gm protein (Diet B), 1200 Cal. and 5 gm protein, or 600 Cal. and 5 gm protein (Diet C), were employed.

2. In no instance did the addition of 3 gm of dl-methionine per day to the low-protein diets decrease the urinary nitrogen excretion.

3. Under the conditions employed, methonine alone does not spare body protein, nor is there any evidence of its utilization from values for the nitrogen or sulfur excretion.

4. The nitrogen minimum ("wear and tear quota") cannot be obtained when the Caloric intake is 600 or 1200 Cal. daily although it is obtained when 2000 Cal. per day are given.

EDITORIAL NOTE

The finding reported in this paper that the requirement for methionine in man is lower than that for the dog confirms the work of Cox et al. also published in the current issue of this Journal. As a matter of fact the present paper was submitted for publication before the one by Cox et al. but its publication was delayed in an attempt to secure simultaneous publication of several papers by different authors representing work undertaken in cooperation with the Research and Development Branch, Military Planning Division of the Office of the Quartermaster General, U.S.A.

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AMINO ACIDS IN NITROGEN METABOLISM WITH PARTICULAR REFERENCE TO THE ROLE OF METHIONINE [1]

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FOUR FIGURES

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Since the time of Folin ('05), the quantities of nitrogen and creatinine excreted in the urine during a period of protein deprivation have been used as a measure of "endogenous" metabolism. Data accumulated at the Iowa State College (Willman et al., '45) indicate that if the albino rat is fed a ration low in nitrogenous constituents but otherwise adequate, the nitrogen metabolism after its initial reduction proceeds at a fairly constant rate. Observations were made in 2 metabolism periods, each 7 days long, after the animals had subsisted for 11 days on the nitrogen-poor diet. However, when whole

[1] Journal paper, no. J-1404 of the Iowa Agricultural Experiment Station, Ames, project no. 799. Preliminary reports appear in the Proceedings of the Federation of American Societies for Experimental Biology, vol. 4, no. 1, 1945, and vol. 5, no. 1, 1946. The data reported are taken in large part from a thesis submitted by Miriam Brush to the Graduate College of the Iowa State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Nutrition, July, 1946. The subject matter of this paper was undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the War Department. The project has been supported in part by a grant from the Poultry and Egg National Board. This aid is gratefully acknowledged.

[2] Evaporated Milk Association fellow of the American Home Economics Association, 1945–1946.

eggs equivalent to 3.5% of protein supplemented the ration in the second metabolism period, the quantity of nitrogen eliminated in the urine was about one-third less than in the previous nitrogen-low feeding period. The excretion of creatinine was similarly altered. The depression of the so-called endogenous metabolism by the simple addition of food proteins to the basal diet casts doubt on the validity of the distinction made by Folin between endogenous and exogenous metabolism, and gives weight from another experimental approach to recent concepts of the fluidity of body processes and of the existence of a dynamic relation between food proteins and tissue proteins (Madden and Whipple, '40; Schoenheimer, '42).

The nature of the body-sparing action that follows the incorporation of eggs in the basal nitrogen-low diet of rats partially depleted of their reserve proteins has been examined in the present investigation.

The study represents a progressive series of experiments. The first investigation was planned to show whether or not the nitrogenous components were specifically responsible for the reduced excretion of nitrogen that followed the introduction of eggs into the basal ration, a mixture of the 10 essential amino acids replacing the eggs in the test diet. A suggestion of Dr. Samuel Lepkovsky [3] together with certain reports in the literature (Miller, '44; Croft and Peters, '45) that methionine may play an outstandingly important role in nitrogen metabolism led to a measurement of its influence by feeding it as the sole dietary source of nitrogen and by omitting it from the mixture of the 10 essential amino acids fed in Experiment I. The discovery that methionine was as effective as were egg proteins in depressing the urinary excretion of nitrogen formed the basis of the remaining experiments.

To determine whether or not methionine was unique in its nitrogen-sparing property, the other essential amino acids were then tested individually.

[3] Committee on Food Research, Quartermaster Food and Container Institute for the Armed Forces.

Since the quantity of nitrogen appearing in the urine of rats does not increase progressively as the quantity of egg proteins is increased (Swanson et al., '47), the response of animals to supplementary methionine offered at graded levels was investigated in the fourth experiment.

In an attempt to elucidate the nature of the physiological action of methionine, the effect of the introduction of cystine and choline into the basal ration was measured. In the last unit of the investigation, whole carcasses of animals as well as hepatic and muscular tissues were analyzed for their contents of nitrogen and methionine in an attempt to gain some information as to the utilization of nitrogen under the various experimental conditions imposed.

In the first 5 experiments, data obtained in the classical nitrogen balance test as developed by Mitchell ('24) and standardized in this laboratory (Swanson et al., '47) were used to measure the response of the experimental animals to the various dietary regimes. In the sixth experiment, the tissues examined were those of rats treated exactly like the animals in the balance experiments and sacrificed at appropriate intervals in the test.

EXPERIMENTAL PROCEDURE

The balance test

Male albino rats, approximately 6 months old, were used in the experiments. They were of Wistar stock, strain A, inbred by brother and sister matings for 94 generations. They had been reared on a stock diet which was a modification of one proposed by Steenbock in 1923 and known in this laboratory as Steenbock V (Swanson et al., '47). The various experimental groups in the first 5 experiments each contained 6 rats. Data pertaining to any rat were discarded in the final analyses only if the animal had failed to eat normally or if some acute infection had developed. This procedure was necessary only in the groups fed valine and arginine in experiment 3, the final number of rats in these groups being 2 and 4, respectively.

The effectiveness of a substance in altering the excretion of urinary nitrogen was determined by comparing the metabolic data obtained during a period when the nitrogen-low diet was fed with those secured in a subsequent interval when this basal ration was supplemented by some source of nitrogen. The test which required 29 days for completion was divided into the following periods: I. Nitrogen-low feeding period: (A) Preliminary depletion period, 11 days, (B) Collection period, 7 days; and II. Nitrogen-feeding period: (A) Adjustment period, 4 days, (B) Collection period, 7 days.

The percentage composition of the basal low-nitrogen diet containing approximately 0.6 mg of nitrogen per gm was as follows: dextrin, 73; butterfat, 10; lard, 10; Osborne and Mendel salt mixture, 4; Ruffex,[4] 2; and NaCl, 1. The ration also supplied daily, 50 mg of cod liver oil, 100 mg of rice bran polish extract, 40 μg of thiamine, 60 μg of riboflavin, 40 μg of pyridoxine, 10 mg of inositol, 10 mg of p-amino benzoic acid, 0.5 mg of nicotinic acid, 0.1 mg of calcium pantothenate, 1 mg of ascorbic acid, 5 mg of choline, 1 μg of biotin, and 0.75 mg of α-tocopherol.

The standardization of the test is described in detail elsewhere (Swanson et al., '47). Preliminary tests showed that the plane of nitrogenous metabolism had stabilized before either of the 2 collection periods was initiated. Also, points such as caloric requirements of the animals, quantity of food ingested and techniques for feeding and for collection of metabolic materials were all considered and controlled.

The animals were weighed daily in each metabolism period. The weights of the rats as recorded in table 1 represent averages of the daily weights of the animals in the 7-day interval. The data also permitted a study of the *change* in body weight that occurred from the beginning to the end of each metabolism period (table 2.) The later calculations were based on data obtained on the second and seventh days of a collection period

[4] Processed rice hulls consisting of alpha cellulose (70%), simple and hydrocelluloses; protein, fat, and vitamin-free; purchased from Fisher Scientific Co., Pittsburgh.

because the withdrawal of food necessary in shifting from 1 period of the metabolism test to another was reflected in the weight of the animal.

Evaluations have been made in terms of the relative quantities of urinary nitrogen excreted in the 2 collection periods and of "body nitrogen spared." The last index is the difference between the nitrogen balances in the 2 test periods, and represents the nitrogen that would have been lost from the body of the animal had no supplementary source of dietary nitrogen been offered in the second metabolism period.

Tissue analysis

In this phase of the experiment, 2 groups of animals were used. The first received the nitrogenous supplement at the end of the first collection period in the 29-day balance test; the second group did not. The animals were sacrificed at appropriate intervals and their tissues analyzed.

Preparation of carcasses. The animals were anesthetized with ether, after which the intestinal tract was excised and washed free of all materials. The entire carcass was then sealed in a tin container and quick-frozen at — 40°C. Each carcass was stored at — 10°C. until time of analysis when it was ground and transferred quantitatively to a flask containing 400 ml of 20% HCl. The mixture was autoclaved at 125°C. and 15-lb. pressure for 7 hours. These conditions assured complete hydrolysis of proteins without destruction of methionine. The solution was diluted quantitatively to volume and samples of appropriate size analyzed for nitrogen and methionine.

Preparation of hepatic and muscular tissues. The rats were starved for 10 hours prior to the time of killing. Before removal of the liver, the animals were partially bled. The liver was divided into 2 portions. About 1 gm was used for the determination of fat and moisture. The remainder was hydrolyzed in acid (4 ml of 20% HCl and 10 ml of water) at 125°C. and 15-lb. pressure.

The gastrocnemius muscles were extirpated carefully, the left was used for the determination of fat and moisture while the right was analyzed for methionine.

Analytical methods. In the estimation of methionine, aliquots of the acid digests were shaken first with small portions of activated charcoal and heated to 60°C. to remove highly colored degradation products (Lavine, '43). The filtrates were analyzed by the method described by Albanese, Frankston and Irby ('44). Interference by cystine and tryptophane was successfully controlled. Analyses were reproducible in the hands of the senior author; constant values were obtained with varying periods of autoclaving and recoveries were satisfactory (99.2%) when known quantities of crystalline methionine were added to egg proteins before autoclaving.

RESULTS

Experiment I

Egg proteins vs. the essential amino acids

In determining the role of nitrogenous constituents in the body-sparing action of dried whole eggs, 3 groups of animals were used. Group I, the negative control series, received the basal low-nitrogen diet throughout the entire experimental period of 29 days. Group II, in the second balance period, was given egg proteins equivalent to 3.5% of the quantity of ration they consumed in the first collection period. The dehydrated eggs were fed apart from the basal ration and supplied 60 mg of nitrogen daily. In the second metabolism period, Group III received 52 mg of available [5] nitrogen daily from a mixture of the 10 essential amino acids. Each acid supplied one-tenth of the total nitrogen of the mixture.

Data describing the response of the rats to the 3 dietary regimes are shown in table 1. Under the conditions of the experiment, a close relationship exists between the quantity of

[5] Those amino acids available only in the *dl-* form were incorporated in the mixture in quantities based on the relative availability of the *d* and *l* forms as reported by Sahyun ('44).

TABLE 1

Average nitrogen metabolism of rats partially depleted of bodily reserves of protein before (Period I) and after (Period II) the inclusion of a nitrogenous supplement in the basal protein-free ration.

DIETARY SUPPLEMENT IN PERIOD II	AV. BODY WT. IN PERIOD		AV. CAL. EATEN IN PERIOD		URINARY N IN PERIOD		N BALANCE IN PERIOD		BODY N SPARED
	I	II	I	II	I	II	I	II	
mg nitrogen/day	*gm*	*gm*	*per day*	*per day*	*mg*	*mg*	*mg*	*mg*	*mg*
Exp. I. Egg proteins vs. the essential amino acids.[1, 2]									
Basal diet: 0	272	254	57	54	260	244	—345	—333	12
Whole eggs: 60	286	284	55	55	275	212	—384	+ 77	461
Ten Ess. A.A.: 54	240	229	46	42	319	288	—401	+ 35	436
Exp. II. Role of methionine[3] in nitrogen metabolism									
Methionine: 3	213	195	50	45	236	149	—347	—210	137
Methionine: 4	257	239	50	47	278	200	—374	—248	126
Methionine: 4	273	248	45	42	332	245	—454	—308	146
Ess. A.A.-Me: 37	260	238	46	41	308	418	...	...	...
Exp. III. Methionine vs. other essential amino acids									
Methionine: 4	257	239	50	47	278	200	—374	—248	126
Arginine: 4	245	227	54	41	259	211	—353	—262	91
Isoleucine: 4	262	243	53	50	276	231	—363	—301	62
Histidine: 4	271	248	56	49	274	236	—373	—298	75
Threonine: 4	276	260	53	49	249	220	—351	—294	57
Leucine: 4	237	224	49	43	280	264	—392	—309	83
Valine: 4	280	263	59	55	307	294	—429	—390	39
Lysine: 4	248	230	52	48	259	251	—365	—301	64
Phenylalanine: 4	268	248	55	48	266	267	—371	—420	—49
Tryptophane: 4	264	244	53	47	269	288	—357	—328	29
Exp. IV. Graded doses of methionine									
Methionine: 1	262	246	52	48	334	202	—462	—293	169
Methionine: 2	255	239	47	45	332	214	—438	—270	168
Methionine: 3	213	195	50	45	236	149	—347	—210	137
Methionine: 4	273	248	45	42	332	245	—454	—308	146
Methionine: 8	261	241	48	46	317	244	—441	—285	156
Methionine: 16	270	250	57	49	332	281	—480	—266	214
Egg proteins: 15	255	241	47	45	298	216	—415	—221	194
Egg proteins: 30	256	242	48	39	309	236	—426	—135	291
Egg proteins: 43	241	233	45	43	294	216	—427	— 44	383
Egg proteins: 59	249	242	43	39	301	228	—421	— 13	408
Egg proteins: 72	261	256	45	39	292	289	—410	+ 70	480
Exp. V. Physiologically essential groups in methionine									
Methionine: 4	257	239	50	47	278	200	—374	—248	126
Cystine: 4	241	224	51	47	256	197	—338	—259	79
Choline: 4	233	217	48	45	257	221	—401	—276	125

[1] Essential amino acids abbreviated as "Ess. A.A." in column I.

[2] Methionine, isoleucine, threonine, valine, and phenylalanine were fed as the *dl*-acids. The nitrogen content of the daily dose of *dl*-threonine and *dl*-isoleucine was 8 mg.

[3] Methionine abbreviated as "Me" in column I.

nitrogen excreted when a diet poor in nitrogenous constituents is fed and the body weight of the animal (Swanson et al., '47). Therefore, the data also have been expressed graphically in terms of body surface area.

The difference in the quantity of urinary nitrogen excreted in the 2 collection periods by the negative control group probably is not significant because the mean excretion per 100 gm of body weight in each of the 2 periods of low-nitrogen feeding was 97 mg. Similar results have been obtained in 5 other experiments (Swanson et al., '47). These data show that any effects associated with the administration of the nitrogenous supplement do not reflect a concommitant change in the plane of nitrogen metabolism.

Also, other experiments were designed to permit a comparison in period II of the metabolism in the last 3 days of the adjustment period with that in the first 3 days of collection. It was found in 4 different studies that the average total nitrogen excreted (mg), respectively, in the adjustment and in the first 3 days of collection period II were: 95, 90; 117, 126; 94, 98; and 107, 108. Apparently then, the adjustment period used in the present series of experiments is long enough for the stabilization of nitrogen excretion after the nitrogenous supplement is added to the diet.

The data obtained in the present experiment, therefore, clearly show that eggs and a mixture of the 10 essential amino acids have the common property of depressing the nitrogenous excretion characteristic of the protein-free feeding period (fig. 1). The feeding of each, likewise, shifts the negative nitrogen balance of period I. When this change is expressed as "body nitrogen spared," both supplements are equally effective in their saving action on body proteins (fig. 1). The picture justifies the conclusion that the marked effects induced by the incorporation of eggs in the nitrogen-poor ration may be ascribed to their amino acid content.

It is interesting to speculate on the significance of the concept, body nitrogen spared, as used herein. It has been possible to correlate the actual loss in body weight incurred from

the second through the last day of the nitrogen-low collection period with the quantity of body tissue represented by the negative nitrogen balance in the same period. Analyses have shown that the average nitrogen content of the carcasses of 36 rats is 2.6%. This figure was used to convert the value for

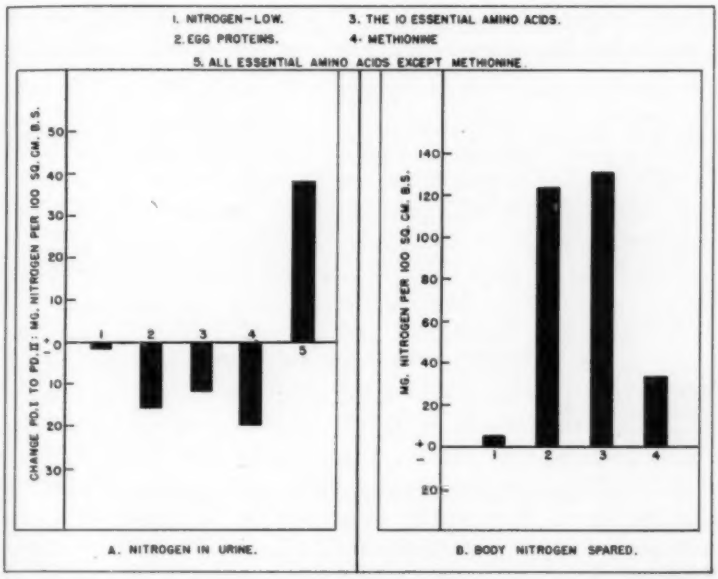
1. NITROGEN-LOW.
2. EGG PROTEINS.
3. THE 10 ESSENTIAL AMINO ACIDS.
4. METHIONINE
5. ALL ESSENTIAL AMINO ACIDS EXCEPT METHIONINE.
CHANGE PD.1 TO PD.2: MG. NITROGEN PER 100 SQ. CM. B.S.
MG. NITROGEN PER 100 SQ. CM. B.S.
A. NITROGEN IN URINE.
B. BODY NITROGEN SPARED.

Fig. 1 Nitrogen metabolism when egg proteins, a mixture of the 10 essential amino acids, methionine, or a mixture of the 10 essential amino acids complete except for methionine supplement a basal nitrogen-low ration fed to rats.

the nitrogen balance, reduced [6] by one-seventh to correspond with the 6-day period over which the actual weight loss was observed, to its equivalent in body tissue. The observed average loss in body weight of the 26 groups of rats used in this study during the first metabolism period was 10 gm; that of the calculated value, 11 gm.

[6] A procedure necessary because food had been restricted in the first day of the 7-day collection period.

The same type of reasoning may be extended to the data obtained when the rats received a nitrogenous supplement in period II, if a basic assumption is accepted, *i.e.*, that if no dietary addition had been made, the rats would have continued to lose weight at the same rate as they had in period I. Such loss of weight occurred in the negative control group of the present experiment as well as in 4 other comparable experiments when the nitrogen-low diet was fed in both metabolism

TABLE 2

Body tissue equivalent of body nitrogen spared when some nitrogenous supplement is added to the protein-free ration of rats partially depleted of bodily reserves of protein.

ADDITION TO BASAL RATION IN PERIOD II	CHANGES IN WT. FROM 2ND TO LAST DAY OF PERIOD		BODY WT. LOSS PREVENTED BY SUPPLEMENT	BODY N SPARED (× 6.25)	TISSUE EQUIVALENT OF BODY N SPARED
	I	II			
mg nitrogen/day	*gm*	*gm*	*gm*	*mg*	*gm*
Exp. I. Egg proteins vs. the essential amino acids					
Basal diet only: 0	— 8	—8	0	10	0
Whole eggs: 60	—11	+3	14	395	15
10 essential amino acids: 54	— 9	+6	15	374	14
Exp. II. Role of methionine in nitrogen metabolism					
Methionine: 3	—13	—5	8	117	5
Methionine: 4	— 7	—7	0	108	4
Methionine: 4	—12	—6	6	125	5
Average	—11	—6	5	117	5

periods, the average loss in weight of 23 rats being 9 gm in period I and 8 gm in period II. On this basis then, both egg proteins and the mixture of the amino acids not only prevented the substantial weight loss (— 8 gm) that would have been induced by feeding the low-nitrogen diet but caused a definite gain in weight from the beginning to the end of the 6-day interval (table 2). Theoretically, the loss in weight *prevented* by the supplement plus the actual increment should be equivalent to the body tissue represented by *body nitrogen spared*, *i.e.*, the difference between the nitrogen balances in

the 2 periods. That such is the case is shown by the data presented in table 2.

Experiment II

The role of methionine in nitrogen metabolism

The effect of the addition of methionine to the basal ration was checked in 3 different experiments. Supplementary methionine equivalent to 3 mg of nitrogen was fed daily in 1 test; to 4 mg, in the other 2 tests. In each experiment, a marked and nearly identical depression in the amount of urinary nitrogen followed the introduction of the supplement (table 1). The effect was as pronounced as that induced by the addition of egg in spite of the fact that the quantities of nitrogen furnished by the 2 supplements were of different order, *i.e.*, 60 mg *vs.* 3 or 4 mg per day (fig. 1). Allison and his co-workers ('45) have also observed that the dog, if adequately depleted of its labile stores of nitrogen, responds similarly to the administration of methionine. It is important to note, however, that while methionine has some ability to spare body tissue (fig. 1), it is much less effective than either egg proteins or the mixture of amino acids. This is not a surprising observation since the metabolic requirements of the body certainly must be far too varied to permit their satisfaction by any one amino acid. The data indicate, also, that deductions as to the body sparing effect of any nitrogenous dietary material should not be made in reference to a reduced excretion of nitrogen in the urine only. This may give not only a partial, but a distorted picture.

The data depict another interesting phenomenon, *i.e.*, the specific capacity of a single substance to reduce losses of nitrogen in the urine concurrent with losses in body weight (table 2). In this instance, however, losses were reduced in magnitude. For example, the 3 groups of rats used in this experiment, on the average, lost 11 gm from the second to the last day of the first metabolism period, and 6 gm in the same interval in period II when methionine was fed. Again, on

the assumption that losses would have been of the same order in the second period as in the first had no methionine been fed, its addition to the diet prevented a loss in weight of 5 gm. Conversion of body nitrogen spared by the incorporation of methionine into the ration to its equivalent in body tissues shows that 5 gm of body weight was spared, a figure identical with the observed weight loss prevented (table 2).

The importance of dietary methionine in sparing body tissue was also demonstrated by observing the effect of its removal from the mixture of the 10 essential amino acids. Its omission caused an increase in the urinary excretion of nitrogen of 110 mg as compared with the decrease of 31 mg which followed the feeding of the complete amino acid mixture. Some of the incomplete mixture was none-the-less utilized since of the 259 mg of available nitrogen fed over the 7-day period, 149 mg did not appear as extra nitrogen in the urine. In a similar experiment, Robscheit-Robbins and Miller ('46) found that the administration of an amino acid mixture complete except for methionine caused an increase in the amount of nitrogen excreted in the urine.

Data in table 3 show that methionine behaves like egg proteins in decreasing the excretion of creatinine.

TABLE 3

Urinary excretion of creatinine when the basal low-nitrogen diet is fed (Period I) and when the same diet is supplemented with dried whole eggs or methionine (Period II).

ADDITION TO BASAL RATION IN PERIOD II	CREATININE EXCRETED IN THE 7-DAY COLLECTION PERIOD		
	Period I	Period II	Decrease in excretion
mg nitrogen/day	*mg*	*mg*	*mg*
Egg proteins: 60	106	82	24
Methionine: 4	97	70	27

Experiment III

Methionine vs. other essential amino acids

In testing the relative effectiveness of the amino acids considered essential for the nutrition of the rat, each was offered

in a dose that supplied 4 mg of available nitrogen per day, a quantity suggested by the work of Wolf and Corley ('39).

Data presented in table 1 and figure 2 show that methionine was outstanding in its ability to decrease urinary losses of nitrogen. The other amino acids depressed its excretion in varying degree.

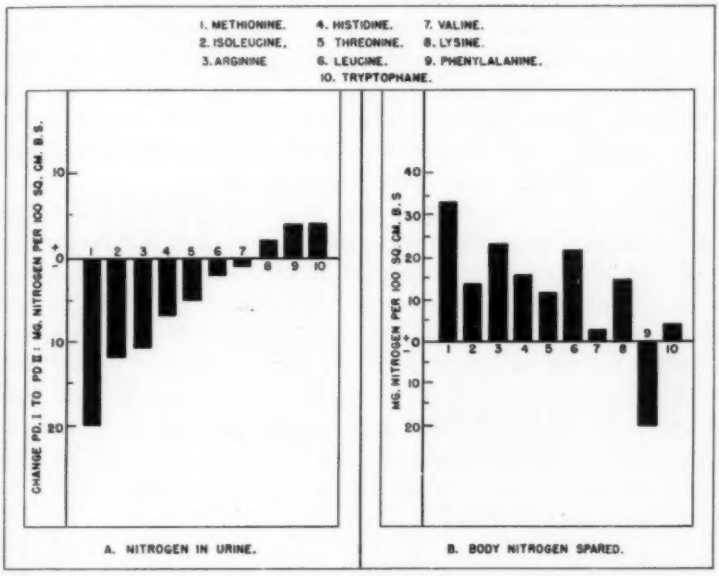

Fig. 2 Nitrogen metabolism when each of the 10 essential amino acids supplements a basal nitrogen-low ration fed to rats.

The data are even more interesting when translated in terms of body-sparing action (fig. 2). Why phenylalanine, valine, and tryptophane are the least effective members of the group is difficult to explain. Indeed, phenylalanine seems to stimulate the breakdown of body tissue, a finding that suggests that the feeding of the acid may lead to the synthesis of the related amino acid, di-iodotyrosine. Unavoidable reduction of the number of animals in the valine group to 2 rats may be reflected in the results obtained from feeding this acid.

The appearance of a bright yellow pigment in the urine of rats fed tryptophane is further evidence of the difficulty the animals experienced in utilizing this amino acid under the conditions of the experiment. The substance excreted, in this instance, was not xanthurenic acid, a compound that may appear in the urines of pyridoxine-deficient rats fed tryptophane (Lepkovsky, Roboz and Haagen-Smit, '45).

It is noteworthy that the sum of the quantities of body nitrogen spared by each amino acid when fed singly was 476 mg while the 10 amino acids fed as a mixture spared 463 mg (table 1). From this it seems that the various amino acids have specific roles in protein metabolism which they fulfill whether fed singly or as part of a mixture.

Differences in the results obtained in this study and those in a similar experiment reported by Burroughs, Burroughs and Mitchell in 1940 may be explained, it seems to the present authors, in terms of relative protein stores, the constancy of the plane of nitrogen metabolism in the period of low-protein feeding, the lag or hangover in the effect of proteins or amino acids on the excretion of nitrogen as observed in rats and dogs in subsequent periods of nitrogen deprivation (Swanson et al., '47; Allison, Anderson and Seeley, '45) and of the repeated use of the same animals without realimentation in successive experiments.

Experiment IV

Effect of feeding graded doses of supplementary methionine

Unlike the progressive increases in the quantity of nitrogen excreted in the urine that accompany graded increases in the quantity of nitrogen fed when many proteins are added to the basal diet, egg proteins maintain the depression at about the same point no matter whether 1, 2, 3, or 4% of egg proteins are furnished in the ration (fig. 3). Only when the diet contains dried eggs equivalent to 5% of protein does the excretion of nitrogen equal that in period I.

Data showing the effect of methionine when fed in quantities that supplied nitrogen ranging from 1 to 16 mg daily are presented in table 1 and may be compared with that of eggs in figure 3. Care should be exercised in comparing the graphic presentations since weight of methionine nitrogen in 1 case, and per cent of egg proteins in the other, were used as the bases for pictorial arrangement. Trends, however, are clear.

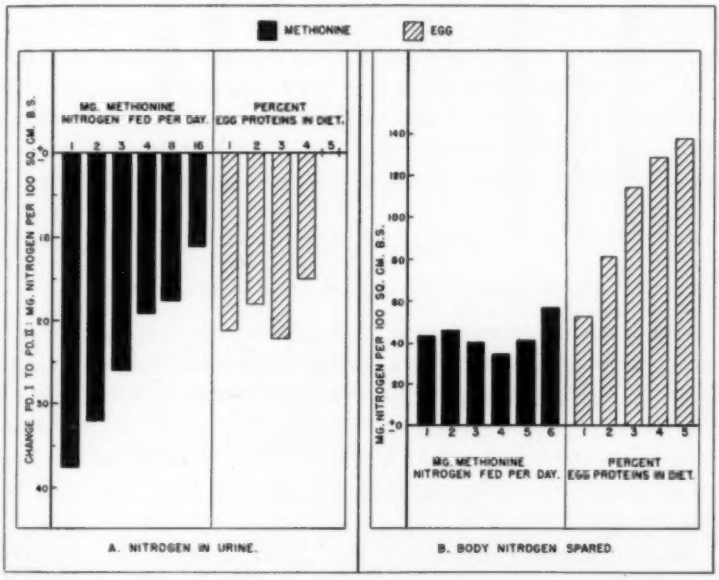
METHIONINE
EGG
MG. METHIONINE NITROGEN FED PER DAY.
PERCENT EGG PROTEINS IN DIET.
CHANGE PD. I TO PD. II: MG. NITROGEN PER 100 SQ. CM. B.S.
A. NITROGEN IN URINE.
MG. NITROGEN PER 100 SQ. CM. B.S.
MG. METHIONINE NITROGEN FED PER DAY.
PERCENT EGG PROTEINS IN DIET.
B. BODY NITROGEN SPARED.

Fig. 3 Nitrogen metabolism when increasing quantities of either methionine or egg proteins supplement a basal nitrogen-low ration fed to rats.

When methionine was offered at the 6 ascending levels of dietary intake, there was a progressive decrease in the depression in the excretion of urinary nitrogen that ranged from 35 to 11 mg per 100 cm^2 of body surface. The data suggest that, under these experimental conditions, the methionine requirement of a rat weighing about 260 gm is 11 mg or less per day. If any excess beyond the amount required is excreted,

the urinary excretions of nitrogen characteristic of the higher levels of intake may be reasonably explained.

Increased quantities of egg proteins in the diet were associated with an increased sparing of body nitrogen (fig. 3). On the other hand, no real difference existed in the respective quantities of body nitrogen spared at each level of methionine intake as shown by analysis of variance. If the requirement is of the low order postulated, it is logical that, however large the quantities of methionine ingested, no additional body nitrogen would be spared, once the requirement is satisfied. Egg proteins, on the other hand, contain substances in addition to methionine, and as the amount of egg ingested is increased, amino acids are provided that also are needed for maintenance or for the synthesis of functional metabolites.

Experiment V

Physiologically essential groups in methionine

Is methionine important by virtue of its organic sulphur, its methyl group or both? In a comparative test, sulphur in the form of cystine and potential methyl group in the form of choline were fed as dietary supplements in quantities that were equivalent to 4 mg of nitrogen. The quantity of choline given was in addition to the amount routinely supplied in the vitamin mixture.

Data presented in table 1 and figure 4 show that cystine and choline apparently share with methionine the ability to decrease urinary losses of nitrogen and to spare body tissue. Any apparent differences are statistically insignificant. This finding agrees with the work of Mulford and Griffith ('42) which indicates that metabolically the same molecule of methionine cannot be used as a source of both organic sulphur and labile methyl groups. Were the reverse true, one molecule of methionine should be able to spare body nitrogen equivalent to the sum of that spared by cystine and choline individually.

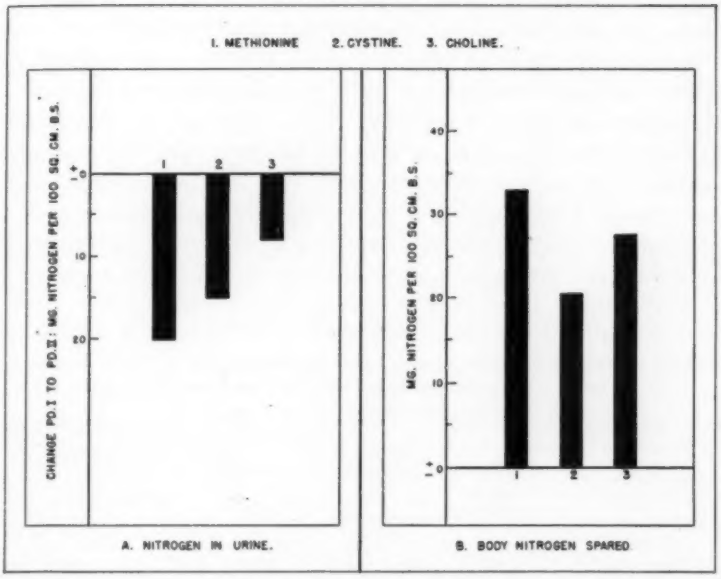

Fig. 4 Nitrogen metabolism when methionine, cystine, or choline supplements the basal nitrogen-low ration fed to rats.

Experiment VI

Tissue analyses

The carcasses of a group of animals removed from the stock colony when 6 months old, of groups fed the ration poor in nitrogenous materials for either 18 or 29 days, respectively, and of another group maintained first on the basal diet for 18 days and then on a nitrogen-supplemented diet for 11 days were analyzed for nitrogen and methionine. Two nitrogenous supplements were fed, *i.e.*, egg equivalent to 42 mg of nitrogen and methionine equivalent to either 1, 2, 4, or 16 mg of nitrogen per day. Data obtained from these groups permitted comparison of total carcass nitrogen at the beginning of the balance test, at the end of period I in the experiment, at the end of the second metabolism period in which a nitrogenous

supplement was ingested, and at the end of the second metabolism period when no supplement had been offered.

Data pertaining to these experiments are presented in table 4. It may be seen that the nitrogen content of the whole carcasses decreased from 8.38 gm to 6.41 gm as the result of feeding the nitrogen-low diet for 18 days. The body weight declined correspondingly. Neither the feeding of the basal diet for an additional 11 days nor the administration of supplementary methionine for the same period had any further effect on the nitrogen content of the carcass. The addition

TABLE 4

Average nitrogen and methionine present in carcasses of rats at different intervals of the balance test following the feeding of either a nitrogen-poor or a nitrogen-supplemented ration in period II.

OBSERVATIONS (3 RATS PER GROUP)	AT BEGINNING OF TEST PERIOD	AFTER N-LOW FEEDING FOR		AFTER 11 DAYS SUPPLEMENTAL FEEDING FOLLOWING 18 DAYS ON N-LOW DIET				
		18 days	29 days	42 mg egg N per day	Mg Me N per day			
					1	2	4	16
Av. body wt., gm	326	253	249	289 [1]	233	226	235	225
Total N, gm	8.38	6.41	6.69	7.75	6.67	6.25	5.52	6.37
N, gm %	2.58	2.52	2.68	2.69	2.86	2.77	2.36	2.82
Total Me, mg	182	132	132	159	144	123	123	145
Me, mg %	56	52	53	55	62	54	51	63
$\frac{\text{Methionine N}}{\text{Total N}}$, %	2.2	2.1	2.0	2.1	2.2	2.0	2.2	2.2

[1] The rats in this group were large, weighing 296 gm after the feeding of a low-nitrogen ration for 18 days.

of egg proteins to the diet for 11 days, however, brought the nitrogen content of the carcass to 7.75 gm.

The total methionine content of the carcass fell during the first 18 days that the nitrogen-low diet was fed, and the data seem to indicate that no further changes were induced thereafter, regardless of whether the animal was continued on the ration poor in nitrogen or whether it was given the basal diet supplemented with methionine. Apparently the body sparing action of methionine (fig. 4) is not related to a gain in either the total nitrogen or the methionine content of the

carcass. This observation is in accord with the relatively small amount seemingly required by the adult rat.

The findings suggest that methionine either serves in the fabrication of essential metabolites contributing little nitrogen to the total value of the carcass or acts as a catalytic agent governing essential processes. After the basal need is met, the methionine may then be used in the regeneration of body tissues providing the other building stones are present, as indicated by the fact that the methionine content of whole carcasses of rats fed egg proteins is higher than that of those fed the basal diet alone or the basal ration supplemented with methionine. The ratio of methionine nitrogen of

TABLE 5

Average weight, total nitrogen content, and methionine content of livers extirpated from rats at end of each period of the balance test, 43 mg of methionine supplementing the ration in period II.

NO. OF RATS	BODY WT.	WT. OF FRESH LIVER	WT. OF DRY FAT-FREE LIVER	TOTAL NITROGEN IN LIVER	NITROGEN IN DRY FAT-FREE LIVER	TOTAL METHIONINE IN LIVER	METHIONINE N / TOTAL N
	gm	*gm*	*gm*	*mg*	%	*mg*	%
N-low diet for 18 days							
4	267	6.41	1.50	127	8.46	41	3.00
N-low diet for 18 days followed by methionine supplementation for 11 days							
5	290	6.96	1.47	154	10.4	50	2.94

the carcass to its total nitrogen content remained unchanged, *i.e.*, 2.1, in spite of any dietary manipulation imposed.

Methionine feeding for the last 11 days of the metabolism test apparently is associated with the elaboration of some hepatic tissue. Average weights of the dry fat-free livers of the animals killed at the end of each experimental period were approximately the same. However, the livers of the animals fed the nitrogen-low diet for 29 days contained 127 mg of nitrogen and 41 mg of methionine as against 154 mg and 50 mg, respectively, when their ration contained methionine (table 5).

It may be recalled that the loss of large quantities of nitrogen through the urine during the period of protein deprivation in the metabolism test was accompanied by an equivalent loss in body weight, and that the addition of methionine to the diet although not preventing these losses did decrease their magnitude. In addition, the quantity of methionine present per gm of dry fat-free gastrocnemius muscle was constant in

TABLE 6

Methionine content of the gastrocnemius muscle of rats analyzed at different intervals of the balance test following the feeding of either a nitrogen-poor or a nitrogen-supplemented ration in period II.

INTERVAL OF BALANCE TEST	METHIONINE/GM DRY FAT-FREE MUSCLE
	mg
Beginning of experiment	38
End of period I; N-low diet, 18 days	36
End of period II	
N-low diet, 29 days	37
Egg supplement, 18 days	35
Me supplement, 18 days	
1 mg Me N/day	38
2 mg Me N/day	36
4 mg Me N/day	38
8 mg Me N/day	38
16 mg Me N/day	38

all experimental groups (table 6). These observations suggest that when methionine is not provided in the diet, the animal cannot selectively remove methionine from its tissues to meet its requirement for the amino acid. Instead it breaks down complete protein units to secure this vital substance, and throws away in the urine a large portion of the remaining molecule.

SUMMARY AND CONCLUSIONS

A marked reduction in the quantity of nitrogen excreted in the urine by standardized rats partially depleted of their bodily reserves of protein follows the introduction of whole

eggs into a protein-free ration. This effect may be ascribed to the amino acid content of the supplement. It has been shown that the influence of an equivalent quantity of nitrogen supplied in the form of the 10 essential amino acids is as marked as that of eggs providing 3.5% of dietary protein. Depressions in the excretion of urinary nitrogen lead to a marked sparing of body tissue. The validity of the concept, "body nitrogen spared," has been tested by relating changes in the nitrogen balance in the 2 metabolism periods to changes in body weight.

Cystine, choline, and all of the essential amino acids except phenylalanine, valine, and tryptophane exert some action in sparing body nitrogen. Of the group, methionine is the most powerful in this respect. No single amino acid, however, can be very important quantitatively in sparing body tissue in the partially depleted animal, in view of the complex demands of the body for the synthesis of its structural and functional components. Egg proteins and a mixture of the essential amino acids are much more effective in this respect than is any individual amino acid, as indicated by the quantities of body nitrogen spared and by restoration of weight loss when the nitrogen-poor ration was fed.

Analyses of whole carcasses, liver, and muscle of adult rats suggest that the effect of methionine in nitrogen metabolism is specific. Continued loss in body weight, the lack of change in the total nitrogen content of the carcass, the constancy of the ratio of methionine nitrogen to total nitrogen of the carcass, and the increment in hepatic tissue when methionine is fed to the depleted animal point to the possibility that this amino acid does not act in the general maintenance of body tissues but in the synthesis of functional proteins and important metabolites.

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COVITAMIN STUDIES

VI. EFFECT OF TOCOPHEROL SUPPLEMENTATION ON THE OUTPUT OF VITAMIN A, CAROTENE, AND FAT BY DAIRY COWS [1]

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SIX FIGURES

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After it had been shown that vitamin E spares vitamin A in the economy of the rat (Harris et al., '44; Hickman et al., '44a, b), it seemed to us that the dairy cow would be the best animal for continuing the experiments. The cow derives vitamin A solely from the carotene of her feed, and the efficiency of utilization, judged by the final transfer to the milk (about 3%), is notoriously low. If the vitamin status or general health of the cow — particularly of barn-fed animals in wintertime — could be benefited by extra vitamin E, this simple procedure would ultimately have wide repercussions in human nutrition.

At the start of the project we believed that both carotene and vitamin A would be found in higher concentrations in the milk of animals given vitamin E. Vitamin A has been fed to cows by various experimenters in the hope of achieving the same end (Deuel et al., '42). Accordingly, we included vitamin A and mixed A and E supplementation in our study. Our preconceived objectives were not realized but we found such a significant increase in the production of butterfat and such an improvement in the health of the herds that we continued the experiment with open mind.

[1] Communication no. 102 from the laboratories of Distillation Products, Inc., Rochester, New York.

The technique of using cows on farms in our vicinity proved extraordinarily difficult and it was only after several failures that we began to get trustworthy results. For instance, in our earliest study the farm manager observed that the cows in one of the groups after about 6 weeks of vitamin E supplementation had much better appetites and healthier looking hair and hides. He transferred all the groups to tocopherols without telling us! At another farm the veterinarian gave our experimental animals intramuscular injections of vitamin A, without our knowing it.

After various experiences of this kind, we finally arranged for the use of a herd of 75 registered Brown Swiss cows.[2] We had complete control of the diet and did all supplement feeding, milk sampling and record keeping. The only restriction made by the owners was that we refrain from taking frequent blood samples, with which we had hoped to follow the vitamin concentration in the plasma parallel with that in the milk.

The form in which the vitamin supplements were fed proved to be of considerable importance. Merely placing measured volumes of oil concentrates of vitamin A or E on the feed in the manger was unsatisfactory. Some cows nosed the oily feed aside and refused it entirely while others ate only part of the supplement. Parenteral administration was not desirable because of the relatively poor utilization of fat-soluble vitamins when injected as compared with oral administration. Simple adsorption of the vitamin concentrates on flour or small portions of grain mixtures allowed for easy feeding but the vitamins proved to be unstable exposed in this way. The problem was finally solved by the use of specially manufactured powdered concentrates of vitamins A and E which had been stabilized against destructive oxidation.[3] Measured quantities of these dry vitamin concentrates were spread on

[2] Mr. E. Merrill and Mr. F. Roberts of Forest Farms, Webster, New York, were exceedingly cooperative and helpful in permitting the use of their dairy herd.

[3] "Myvadry," manufactured by Distillation Products, Inc.

the grain mixture once daily. In no instance did the animals refuse to consume the supplements completely.

Milk produced by each cow was carefully weighed at each milking on a Chattillon balance and the amount recorded. It was analyzed for butterfat twice each month, once by the licensed state tester and once in our own laboratory. The official Babcock test was used. Milk samples representative of the day's total output were analyzed every 2 weeks or oftener for vitamin A and carotene by the method of Koehn ('40). Calibration curves were constructed using a standard vitamin A ester concentrate and crystalline β-carotene. Consequently, analytical results were determined in terms of micrograms of vitamin A and of carotene. Although this is the preferable method of reporting results we have converted the values to "international units" to facilitate comparison with published figures. To convert micrograms of vitamin A to international units the conversion factor 3.5 was used. A factor of 1.67 was employed to change micrograms of carotene to international units. The calculation of "4% milk" was made using Gaines' formula ('28) namely "(pounds milk produced $\times$ 0.4) + (pounds fat produced $\times$ 15.0) = pounds 4% milk."

The cows were grouped insofar as possible according to age, stage of lactation, and relative amount of milk produced. They were fed grain throughout the year, even while on pasture from May through August. During the rest of the year they received corn silage and chopped, field-cured hay. Their feed was analyzed occasionally for carotene content and found to vary considerably. As a reasonable average value for vitamin A intake during stall feeding we used 550,000 units per day, of which approximately 40% was furnished by silage, 1% by the grain mixture, and 59% by hay.

Effect of vitamin E on fat concentration of milk and on milk output

It became evident in our preliminary experiments that the cows receiving extra amounts of vitamin E secreted milk with higher fat concentration. This increase was fairly large and

regular. Conversely, the volume of milk secreted by the vitamin E supplemented cows was usually slightly reduced. However, the overall change in production on the basis of "4% milk" was almost invariably an increase. An experiment was therefore set up to test the significance of the increase of

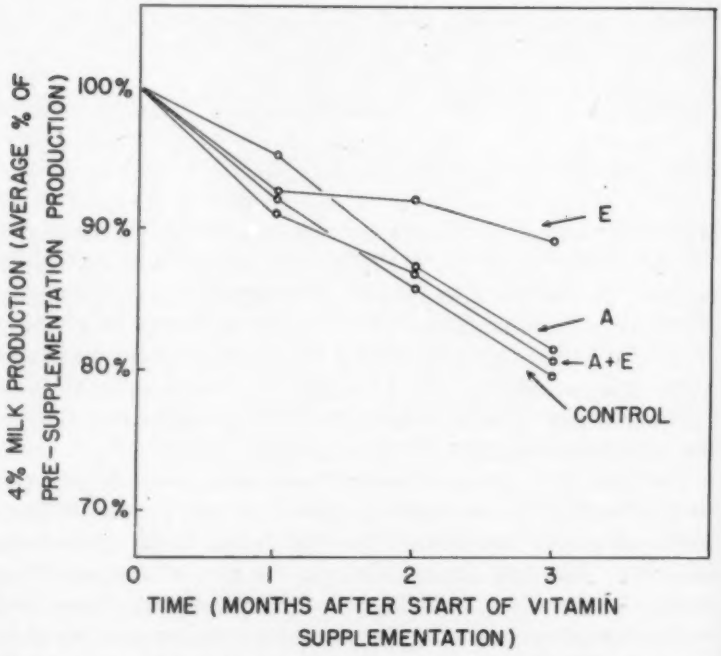

Fig. 1 Effect of vitamins A and E supplementation on the output of 4% milk by matched groups of Brown Swiss dairy cows.

"4% milk" production. Four groups were selected, each containing 4 carefully matched cows, and each group was supplemented in a different way with vitamins as shown in figure 1. The cows receiving vitamin E for more than 1 month of feeding maintained their milk production above the usual rate of decline. Cows receiving vitamin A and, surprisingly, vitamin A plus vitamin E did not show this effect. The cows

in this experiment were not used after 3 months of supplementation because several in each group had passed their ninth month of lactation.

Following this confirmation that vitamin E increased milk and fat output the experiment was repeated using a larger

TABLE 1

Effect of vitamin E supplementation on butterfat concentration.

NO. OF COW	AVERAGE BUTTERFAT % DURING PREVIOUS LACTATION (300 DAYS)	FAT CONCENTRATION OF MILK PRODUCED (%)					
		1[1]	2	3	4	5	6
		Group I control					
1A3	4.14	3.3	3.2	3.6	4.0	3.8	3.5
1B4	4.14	2.7	3.0	2.1	3.2	4.9	2.9
1C3	3.44	3.3	3.2	3.8	3.5	3.7	4.0
1D6	4.38	4.2	3.5	3.8	3.7	3.8	2.3
1E7	3.66	5.0	3.4	2.5	3.5	3.1	3.4
1F1	4.21	2.5	2.8	4.3	3.1	4.0	3.8
1G6	4.38	4.2	3.5	3.8	3.7	3.8	2.3
Monthly average	4.05	3.60	3.23	3.41	3.53	3.87	3.17
Total average		3.47					
		Group IV vitamin E supplement					
4A31	4.44	4.4	4.4	4.2	4.3	4.0	4.3
4B34	4.71	5.0	5.0	4.5	3.7	4.3	4.2
4C30	3.87	4.6	4.7	5.1	4.6	4.3	4.5
4D35	4.35	5.5	4.7	4.7	4.2	4.0	4.0
4E36	3.97	3.6	3.3	3.5	3.6	3.5	3.7
4F34	3.87	5.0	4.5	3.7	4.3	4.2	3.7
4G33	3.87	5.3	5.3	5.2	5.2	5.6	5.4
Monthly average	4.15	4.77	4.56	4.41	4.27	4.27	4.26
Total average		4.42					

[1] Figures refer to calendar months during experiment.

number of matched pairs of cows which were not so far advanced in lactation. One group was used as controls while the animals of the other group were fed 1.0 gm extra of natural mixed tocopherols daily. The findings are shown in tables 1 and 2. Analyses of variance (Bliss and Marks, '39) of these

data confirm the fact that both butterfat concentration and "4% milk" production are significantly ($P = <0.05$) increased by tocopherol supplementation.

TABLE 2

Effect of vitamin E supplementation on output of "4% milk."

NO. OF COW	AVERAGE PRODUCTION DURING PREVIOUS LACTATION (300 DAYS)	PRODUCTION OF "4% MILK" (LBS./DAY)					
		1[1]	2	3	4	5	6
		Group I control					
	(lbs./day)						
1A3	37.62	37.03	36.64	32.19	34.96	30.51	27.48
1B4	30.51	29.57	33.09	24.98	29.90	25.65	20.60
1C8	37.07	37.00	36.60	30.51	27.50	26.47	27.13
1D9	25.61	46.18	42.91	34.40	34.56	30.42	29.85
1E7	36.71	57.73	50.74	38.41	39.90	34.01	31.28
1F1	37.66	36.64	35.47	45.11	31.07	30.84	28.29
1G6	25.61	52.20	44.40	43.70	40.24	34.60	29.50
Monthly average	32.97	42.34	39.98	35.61	34.02	30.36	27.73
Total average	32.97	35.01					
		Group IV vitamin E supplement					
4A31	27.13	42.63	47.93	45.08	42.80	36.52	36.46
4B34	34.04	62.10	59.80	53.79	51.06	44.36	40.59
4C30	21.86	34.34	28.31	31.07	32.34	30.45	30.96
4D35	33.90	61.90	59.51	57.86	54.58	51.38	54.81
4E36	30.93	32.59	34.25	33.91	31.91	31.82	28.83
4F38	34.04	59.80	53.79	50.06	48.36	40.59	36.12
4G39	30.30	41.14	38.40	36.78	34.06	32.78	33.23
Monthly average	30.31	47.79	46.00	44.08	42.16	38.23	37.29
Total average	30.31	42.59					

[1] Figures refer to calendar months during experiment.

It should be pointed out that the experiment from which the data in tables 1 and 2 were obtained was conducted throughout the 1-year period from February, 1945, to February, 1946. The supplements were given during the summer months of pasture feeding as well as during the winter months of stall feeding.

Unexpectedly, the effects of additional tocopherol persisted even through the period of pasture feeding.

In general, the increase in "4% milk" output due to tocopherol supplementation varies from 5 to 15%. However, the increase in fat concentration induced by tocopherol ranges between 10% and 40%. Figure 2 shows the butterfat levels in 3 matched groups of 6 cows each, studied for 21 months. It is evident that the additional tocopherol has induced a metabolic change which is reflected in an increase in fat concentration in the milk. That this effect is rather rapid was

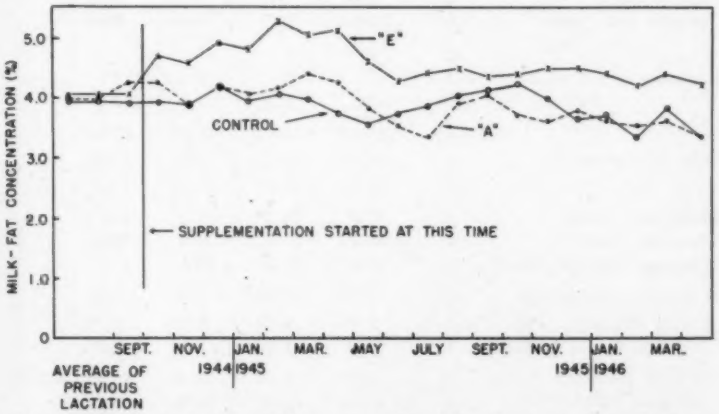

Fig. 2 Changes in fat test of dairy cows induced by vitamin supplementation.

demonstrated by several tests of the cross-over type. Individual cows in the vitamin E supplemented group ceased receiving a supplement, and cows in the control group were given vitamin E. Ten days after the changes were made there were noticeable changes in fat concentration in the milk. Upon returning to the original feeding program the fat concentrations were reversed, again within 10 days.

Further confirmation of this effect of vitamin E on milk-fat production was obtained in a short experiment at the Silver Forest Farm in Forestville, New York.[4] Four closely

[4] We appreciate the kindness of Mr. L. Hawkins and Mr. I. Dodge in allowing us to report these data.

matched trios of registered Guernsey cows were used. The groupings and production data are shown in table 3. Cow 1 in each trio was used as the control and received no supplement. Cow 2 received 1.0 gm of vitamin E daily in the form

TABLE 3

Output of "4% milk" by Guernsey cows as affected by vitamin E supplementation.

	COWS [1]	GROUP OR TRIO NUMBER				AVERAGE
		1	2	3	4	
Date of birth (month and year)	1	12-39	1-39	3-43	6-43	
	2	8-41	1-40	7-42	1-43	
	3	10-42	12-37	6-43	8-42	
Lactation	1	4	4	1	1	
	2	3	4	2	1	
	3	2	6	1	2	
Date of freshening	1	11-45	11-45	11-45	11-45	
	2	12-45	11-45	11-45	10-45	
	3	12-45	11-45	12-45	10-45	
Production during January, 1946 (average lbs. milk/day)	1	47.2	46.2	30.3	40.0	
	2	54.5	57.5	33.2	22.6	
	3	50.8	51.3	32.1	24.8	
Milk-fat concentration during January, 1946 (average % fat in milk)	1	4.30	5.58	5.70	4.97	
	2	5.52	4.68	4.00	4.90	
	3	4.59	4.65	5.26	4.90	
4% milk produced during January, 1946 (lbs.)	1	49.33	57.17	38.09	45.85	
	2	60.95	63.35	33.20	25.70	
	3	55.25	56.37	38.18	28.06	
Change in production of 4% milk, compared with that of Jan., in Feb. (%)	1	—18.0	—26.6	—26.0	—14.1	—21.2
	2	—10.9	—14.3	—14.3	—5.8	—11.3
	3	—9.4	—1.8	—14.2	—9.1	—8.6
Change in March (%)	1	—10.9	—24.8	—26.1	—16.0	—19.5
	2	—7.7	—16.7	—19.2	—4.4	—12.0
	3	—1.7	—12.8	—22.7	—8.5	—11.4
Change in April (%)	1	—23.9	—32.8	—36.8	—20.9	—28.6
	2	—9.6	—20.0	—24.8	—10.0	—16.1
	3	—12.2	—25.1	—23.6	—11.6	—18.1

[1] Cow 1 in each trio received no supplement and was the control.

Cow 2 in each trio received 1.0 gm of mixed tocopherols, of which 10% was in the α- form.

Cow 3 in each trio received 1.0 gm of tocopherols, of which 60% was in the α- form.

of a concentrate of mixed natural tocopherols, chiefly γ- and δ-tocopherol with 10% α-tocopherol. Cow 3 received the same amount of vitamin E in the form of a concentrate of mixed natural tocopherols (60% α-tocopherol) similar to that used at Forest Farms. The supplements were begun February 1, 1946. The production level for January, 1946, for each cow was used as the base value and changes from this constituted the criterion of response due to supplementation. Figure 3

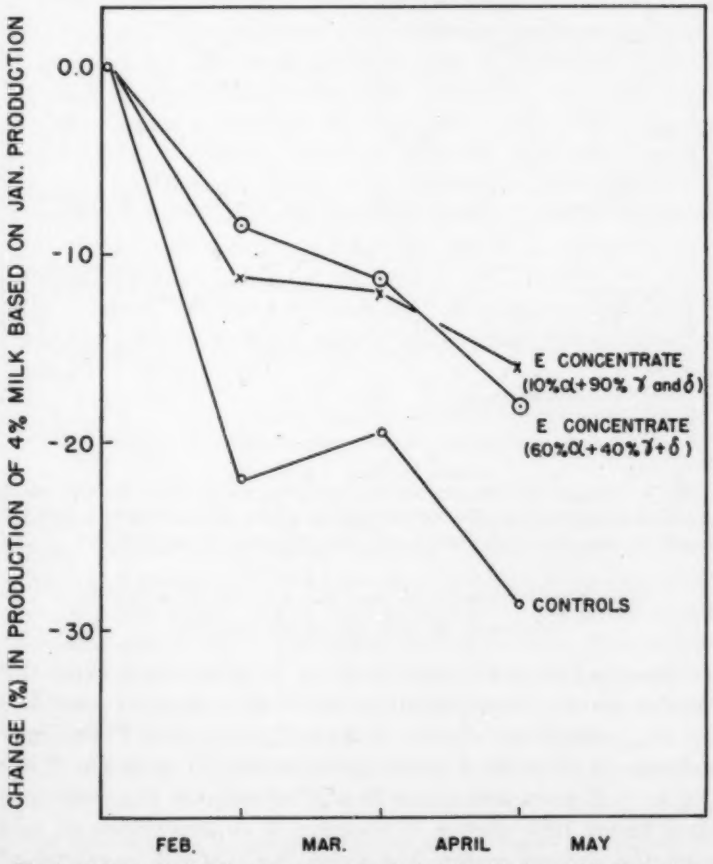

Fig. 3 Effect of vitamin E on the output of 4% milk by matched groups of Guernsey cows.

shows these changes and indicates that: (1) vitamin E beneficially influenced "4% milk" production in Guernsey cows, and (2) the γ- and δ-tocopherol concentrate low in α-tocopherol seems equally effective for this purpose. The differences in "4% milk" production recorded in figure 3 between the control group and the 2 vitamin E supplemented groups were statistically significant ($P = <0.05$).

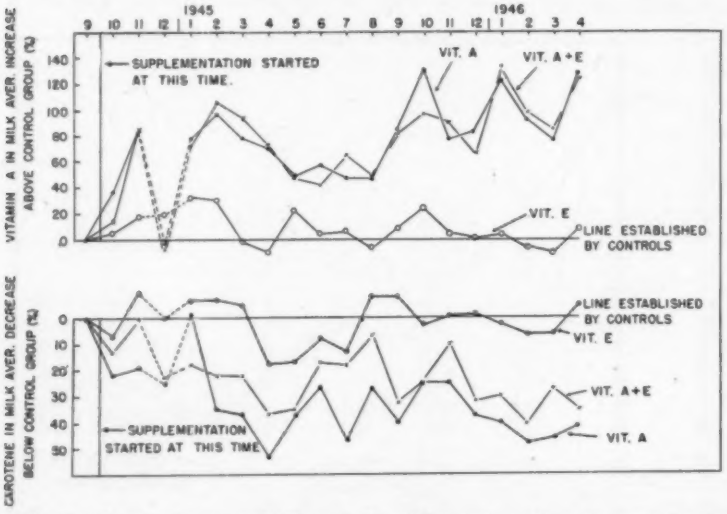

Fig. 4 Changes in concentration of vitamin A and carotene in milk due to vitamin supplementation. The dashed portions of the curves indicate a period of 2 weeks in December, 1944, during which the cows were not supplemented.

Effect of vitamins A and E on concentration of vitamin A and carotene in milk

Data for this are shown in figure 4. It seems obvious that insofar as the concentration or the total output of vtamin A *per se* is concerned, vitamin E caused no increase. The average increase in vitamin A above the controls for both the A and the A + E groups is about 75%. The vitamin E group averaged about 10% higher in vitamin A concentration of milk than the control group, but since the increase occurred all

at the beginning of the period as shown in the curves in figure 4 it is not considered significant.

The effect of vitamin E on carotene concentration in the milk was somewhat different. It is now well established that large doses of vitamin A depress the level of carotene in the blood and milk of dairy cows (Deuel et al., '42). This is confirmed in figure 4 where the carotene level of the vitamin A group is 33% below that of the control group. However, when vitamin E was fed simultaneously with the vitamin A the depression of carotene was not so pronounced; it was about 23% lower on the average than that of the control group. Vitamin E alone did not affect the carotene of the milk. There is rather great variability of response in these experiments, but since the trend is constant and has been confirmed in shorter tests it seems reasonable to conclude that when vitamin E is administered with vitamin A the carotene concentration in the milk is lowered only two-thirds as much as when vitamin A is fed alone.

The overall efficiency of transfer [5] of carotene from feed to vitamin A potency in the milk is shown in figure 5. The formula used is as follows:

$$\text{"Efficiency of transfer"} = \frac{\text{I.U. secreted by supplemented cows} - \text{I.U. secreted by control cows}}{\text{I.U. of A in daily supplement}}$$

The bar graph at the top of figure 5 shows the apparent variation in efficiency of transfer over a 15-month period. First, however, it should be noted that the overall average efficiencies for the A-fed group and the group fed A + E are essentially the same, about 2.4%. The efficiency percentages vary during the year from as much as 6% in the winter (January and February) during stall feeding to less than 1% in the summer (June and July) when fresh pasturage is available.

The absolute values and seasonal variations for the average concentration of vitamin A potency (vitamin A *per se* plus

[5] I.U. secreted equals the sum of the carotene and the vitamin A in international units contained in the milk secreted daily.

carotene) in the milk of the control group are also of interest (fig. 6). For example, the maximum average concentration (approximately 1,800 units per quart) occurs in the period from June through October and rapidly drops to a minimum (800 to 900 units per quart) in the period from January through April. This means, of course, that the milk consumer may obtain milk in the winter which furnishes him with

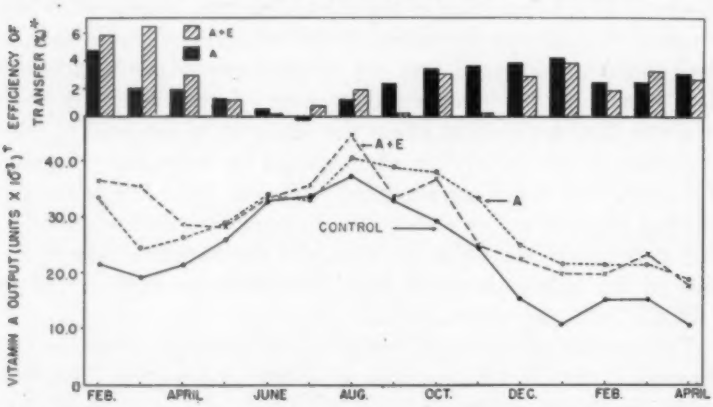

Fig. 5 Influence of vitamin A vs. vitamin A and E in effecting transfer of vitamin A potency into the milk.

† Average number of I.U. of vitamin A potency secreted per day per cow (vitamin A + carotene). Increase in vitamin A potency (vitamin A + carotene) of milk related to vitamin A in daily supplement.

* Increase in vitamin A potency (vitamin A plus carotene) of milk related to vitamin A in daily supplement.

only 50% of the vitamin A potency of summer milk. These variations are also shown in figure 6, expressed on the basis of butterfat. Carotene contributed about 34% of the total vitamin A activity of the milk (Lord, '45). This varied from 25% in the winter to 40% in the summer.

Referring again to figure 5, it is evident that supplementation of the dairy cow with 250,000 units of vitamin A per day during the winter is not enough to maintain the vitamin A potency of the milk at the summer level. Deuel and co-workers ('41) have previously noted this and state that the diet of the

cow must be supplemented with at least 700,000 units of vitamin A to achieve a significant increase in vitamin A concentration in the milk. In experiments not reported in detail in this communication, we have fed vitamin A supplements of 500,000, 750,000, and 1,000,000 units per day. The 2 higher levels were necessary to maintain the vitamin A potency at summer values. This, of course, would not be economical as a means of supplying vitamin A to the consumer. It should be done only if there is evidence that it improves the general health of the cow.

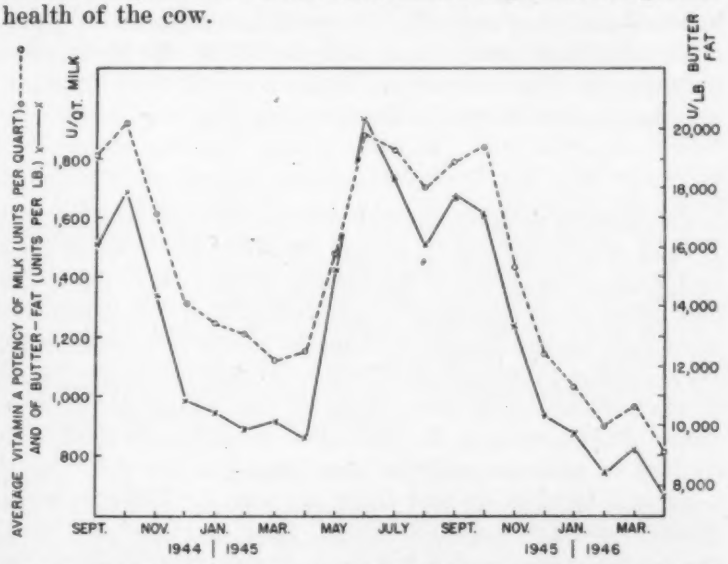

Fig. 6 Changes in total vitamin A potency of milk and of butter fat of Brown Swiss control cows.

General effects of vitamin E supplementation

Impressions and opinions of the herdsman and farm manager, although subjective in nature, are also of considerable value in evaluating the responses to vitamin E supplementation. These men reported that the supplemented cows had increased appetites. They also noted that the cows looked healthier and more vigorous. Generalized infections were

completely absent. There was less mastitis (strip-cup test) than in the controls or in the herds of previous years according to the records. The ratio of services to conceptions decreased from 5 to 1 in previous years to less than 3 to 1 among the supplemented cows. There were no stillbirths and only 1 abortion. On only 1 occasion was it necessary to remove the fetal membrane following parturition. The record for the previous year showed 3 stillbirths, 4 abortions, and retained placentas requiring the services of a veterinarian after the birth of almost every calf. In general, our previous experience with other herds, which indicated that supplementation with vitamin E or with both vitamins A and E is highly desirable, has been confirmed in this investigation.

DISCUSSION

The benefits to dairy cows of supplementation with vitamin E (and A) may be due in part to the effect of these vitamins on the microorganisms of the rumen. Vitamin E in particular may affect the oxidation-reduction potential of rumen contents enough to change the proportions of the various organisms. We considered the possibility of this mode of action of vitamin E upon finding that very little of the administered tocopherol was secreted in the milk. For example, the tocopherol content of summer milk-fat was 42 μg per gm for control cows and between 49 and 53 μg per gm for E-fed animals. Winter milk-fat contained about 23 μg per gm and 47 μg per gm for control and E-fed cows, respectively. Some of the tocopherol apparently found its way into the body of the cow and was secreted into the milk. The efficiency of this transfer is very low; 2% would seem to be a maximum value. However, it should be kept in mind that we do not know the optimum dose of tocopherol for cows. All of these experiments were carried out using 1.0 gm of tocopherol. We have yet to investigate the responses at different dosage levels.

The vitamin E concentration in the blood of the cows was determined only at 1 time of the year, during February. The

average for the control group was 0.45 mg % and for the E-fed group 1.06 mg %. In previous studies on Holstein cows the tocopherol levels in the blood of control and E-fed cows showed the same difference. The tocopherol level considered normal for humans is 1.0 mg or more per 100 ml of plasma (Quaife and Harris, '44), and it is interesting to speculate that perhaps the tocopherol supplement was necessary to raise the vitamin E nutriture of the cow to normal.

An interesting sidelight of this study concerns the difference in curd formation in the milk from the various groups of cows. This will be investigated in detail as soon as possible. In brief, the milk from E-fed cows coagulates less quickly than milk of control cows, while the milk of A-fed cows coagulates more quickly. Coagulation is considered as visible clotting of the protein and separation of the milk into whey and clot. Surprisingly enough, the increase in titratable acidity is the same in all of the milks, but invariably the milk from the A-fed cows separates more quickly and that from the E-fed cows less quickly than that from the control cows. It is not impossible that some fundamental change in the proteins of the milk has taken place under the influence of either vitamin A or vitamin E which would account for the difference in clotting.

SUMMARY AND CONCLUSIONS

A herd of purebred Brown Swiss dairy cows divided into 4 comparable groups has been studied for a period of 20 months. Three groups received daily supplements of: (1) 250,000 units of vitamin A, (2) 1.0 gm of mixed natural tocopherols, and (3) both vitamin A and tocopherols, and a fourth group received no supplement. Milk production, fat concentration in the milk, total fat production, and output of vitamin A and carotene in the milk were studied extensively. Shorter experiments were conducted on the tocopherol content of the blood and milk of the cows.

Tocopherol supplementation increased milk-fat concentration about 27% and total milk production ("4% milk") about

21%. The same response was induced in Guernsey cows fed vitamin E. Only cows in the same stage of lactation and as nearly matched as possible in other respects, such as age and number of lactations, were compared.

Supplementation with vitamins A and E was no better than with vitamin A alone in increasing the vitamin A *per se* in the milk. However, the decrease in carotene concentration in the milk due to the feeding of vitamin A was less severe (approximately 23% below the control level rather than 33%) with the simultaneous administration of vitamin E.

The efficiency of transfer of ingested vitamin A and carotene into vitamin A and carotene in the milk was not increased by supplementation with vitamin E alone. The average value for a 1-year period was 2.4%. This varied from about 6% in the winter under barn-feeding conditions to less than 1% in the summer when fresh pasture was available. The total output of vitamin A potency (vitamin A plus carotene) of the control cows varied from a maximum value in August to 30% of this in January. Carotene contributed about 34% of the total vitamin A activity of the milk, and this varied from 25% in winter to about 40% in summer. General improvement in appearance, reproduction, and health was noticed in the parts of the herd supplemented with vitamin E and vitamins A and E.

Finally, in comparing the benefits to be derived from the 3 types of supplementation, E, A, and E + A, it must be concluded that the major benefit in health and milk output was derived from vitamin E alone. The only obvious result of vitamin A supplementation was to increase the vitamin A content of the milk, this at the partial expense of carotene content. The combined supplementation of E and A allowed the A content of the milk to rise without a pronounced fall of carotene, but the increase in butterfat which we have come to expect from vitamin E supplementation was entirely repressed by the vitamin A. Vitamin E therefore emerges as the preferred supplement to the ordinary diet of dairy cows.

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SOME OBSERVATIONS ON THE NUTRITIONAL VALUE OF DIALYZED WHEY SOLIDS

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Lactalbumin is known to be a more nearly complete protein and in biological studies has proved to be a far more efficient source of dietary nitrogen than casein (Weech, '42; Melnick, Cowgill and Burack, '36; Melnick and Cowgill, '37). It is also known that human milk is much higher in lactalbumin than cow's milk. The reported composition of average human milk varies from about 1.0% lactalbumin and 0.5% casein (ratio 2:1) (Williamson, '44) to about 0.75% lactalbumin and 0.5% casein (ratio 3:2), the latter being the most widely accepted figure (McLester, '39; Jeans, '43). Cows' milk runs fairly constant at about 0.5% lactalbumin and 3.0% casein (ratio 1:6). It would seem logical that an infant food of a similarly high lactalbumin/casein ratio as that of human milk should have a higher nutritional value than one based on cows' milk alone.

A formula of this kind, high in lactalbumin, was devised by the partial substitution of whey for milk proteins. Comparative feeding tests showed unexpectedly, however, that the rats on the normal milk (low lactalbumin) diet gained more on slightly less food intake than the rats on the high lactalbumin diet. This is the reverse of what had been expected. It was the object of this investigation to clear up the apparent discrepancy between the higher biological value of lactalbumin and the poorer results in actual feeding when the lactalbumin is incorporated into the formula by the addition of whey solids.

EXPERIMENTAL

Feeding tests were carried out with male albino rats of the Sprague-Dawley strain, 21 days of age and with a 5-gm weight range. They were placed on individual raised screen cages and fed the experimental diets for 6 weeks. Food and water were given ad libitum and food consumption records were kept for the group as a whole. Six animals were fed each ration. Weekly weight gains were recorded for each rat and the mean as well as standard deviation for the 6-week gains reported in the results. This same procedure was followed throughout all the experiments. The vitamin and mineral supplement was identical in all diets. The protein level in all experiments was held below that for optimal rat growth, viz., at 12% and in this as in all other respects the diets closely approximate the composition of human milk. The data are summarized in tables 1 and 2.

Test no. 1

This was a comparison of a diet based on milk proteins only (Control) with a high lactalbumin diet containing milk plus whey proteins (Test). (Columns 1 and 2 in table 1.)

The increase in rate of growth and the greater efficiency in converting the food into body tissue, both indicate a superior nutritional value of the control formula.

In the control diet the lactalbumin/casein ratio was about 1 : 6 and in the test diet it was 3 : 2 as in human milk. The fat content was identical in both formulae but the carbohydrate was slightly different. Four per cent carbohydrates were withheld from the test diet, to compensate for the high mineral content of the whey (table 2). This slight difference in carbohydrates was not deemed important.

During the experimental period, animals of both groups were normal and healthy in appearance, although, the animals receiving the test ration containing whey had diarrhea throughout the 6-week period.

TABLE 1

Comparative rat feeding tests with milk and dialyzed or undialyzed whey additive.

	TEST I		TEST II			TEST III	
	Milk proteins only	Milk and whey proteins	Undialyzed whey proteins	Dialyzed whey proteins	Dialyzed whey proteins plus Minerals	Milk proteins only	Milk proteins plus dialyzed whey proteins
	Control	Test	Control	Test	Check	Control	Test
	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7
Ration:							
Dry whole cows' milk (gm)	45.30	21.00	21.20	21.20	21.20	45.30	21.00
Undialyzed whey powder (gm)	....	57.70	57.70	....	....	....	....
Dialyzed whey powder (gm)	....	....	....	44.00	44.00	....	44.50
Lactose (gm)	24.32	....	....	9.20	9.20	25.32	....
Sucrose (gm)	13.24	0.20	....	....	....	13.24	13.24
Butterfat (gm)	15.10	21.20	21.10	21.27	21.27	15.10	21.26
Minerals (gm)	0.78 [1]	....	....	....	3.00 [2]	0.78 [1]	....
Lactic acid (gm)	0.59	....	....	.40	.40	0.59	....
Water (gm)	0.67	....	....	3.91	.91	0.67	....
Composition:							
Protein (%)	12.00	12.00	12.05	12.03	12.03	12.00	12.00
Lactalbumin (%)	1.72	7.20	7.20	7.20	7.20	1.72	7.20
Casein (%)	10.28	4.80	4.85	4.83	4.83	10.28	4.80
Carbohydrate (%)	54.51	50.37	50.90	50.90	50.90	54.51	54.51
Lactose (%)	41.27	50.17	50.90	50.90	50.90	41.27	41.27
Sucrose (%)	13.24	0.20	....	....	....	13.24	13.24
Fat (%)	27.50	27.50	27.30	27.30	27.30	27.50	27.50
Minerals (%)	3.44	6.39	6.37	3.37	6.37	3.44	3.44
Lactic acid (%)	0.67	0.67	.99	.99	.99	0.59	0.59
Moisture (%)	0.70	2.70	2.60	5.60	2.60	0.67	1.80
Results:							
Average weight gained (gm)	99.00	91.00	102.00	122.00	102.00	99.00	125.00
Standard deviation (gm)	12.86	12.90	8.66	10.72	6.42	12.86	14.15
Standard deviation (%)	13.00	14.20	8.48	8.78	6.28	13.00	11.32
Daily food consumption (gm)	8.36	9.80	9.43	10.00	10.20	8.36	9.31
Weight of ration necessary for 1 gm gain in body weight (gm)	3.55	4.53	3.88	3.44	4.20	3.55	3.10
Galactose excretion (%)	...	...	12.38	6.83	14.00	18.89	8.48

[1] Osborn-Mendel mixture.

[2] Whey diffusate ash.

To determine the possible influence of the higher mineral content on the above results, it seemed desirable to introduce the lactalbumin into the diet free of minerals or at least with a greatly reduced mineral content. In the customary processes for separating lactalbumin from the other whey constituents, denaturization occurs and the resulting insolubility is undesirable in infant feeding formulae. To remove the minerals without denaturing the protein, a special commercial dialysis technique was worked out which reduced the soluble ash fraction from 5.46% to 1.30%.

Diets in which this dialyzed whey replaced undialyzed whey showed greatly improved growth response. In order to confirm this result, the fraction dialyzed out of the whey (diffusate) was collected, dried, ashed and was then reintroduced to the dialyzed whey to restore the original mineral concentration. On the assumption that the diffusate minerals are the factors responsible for the adverse effect on growth, the result of this test may be expected to be similar to that obtained with undialyzed whey, and this is confirmed by the test.

The ashing eliminates the possibility of an organic growth inhibiting factor being responsible, but without a qualitative and quantitative determination of the various mineral constituents removed by dialysis, the question remains unanswered which of the minerals is responsible for the adverse effect on growth.

Test no. 2

This was a comparison of diets containing undialyzed whey (Control), dialyzed whey (Test) and dialyzed whey with restored minerals (Check) columns 3, 4 and 5 of table 1.

The composition of the diets in all 3 groups is identical with the exception of the minerals.

The beneficial results of dialysis are evident from an inspection of the data. The rats on the dialyzed whey diet ate 6% in excess, and gained 19.6% more than those on undialyzed whey; in all they showed a 12.8% greater gain in body weight

per gm food intake than the rats on the undialyzed whey diet. The slight variation in the results of the control group and the check group may be due to some changes in chemical composition of the minerals brought about by the ashing at high temperature (700°F).

The galactose excretion was determined after 5 weeks on the experimental diet. During the experimental period, animals receiving all 3 diets were normal and healthy in appearance. Diarrhea was prevalent among the rats receiving the test ration only during the first week of experiment. Animals receiving the undialyzed whey ration and those receiving the dialyzed whey plus the ashed whey diffusate had diarrhea throughout the 6-week period.

TABLE 2

Proximate analysis of undialyzed and dialyzed whey.

	VALUES FOUND	
	Spray dried whey powder	Spray dried dialyzed whey powder
Total solids (%)	96.22	97.12
Moisture (%)	3.78	2.88
Ash (%)	8.93	4.93
Protein (N × 6.38) (%)	11.08	14.47
Fat (ether extract) (%)	1.09	1.05
Lactic acid (%)	1.71	1.33
Carbohydrate (by difference) (%)	73.41	75.34
Partition of the ash		
Total ash (%)	8.93	4.93
Insoluble ash (%)	3.47	3.63
Soluble ash (%)	5.46	1.30
Chloride (as Cl) (%)	1.75	0.24
Calcium (as Ca) (%)		0.81
Phosphorus (as P) (%)	...	0.67
Sodium (as Na) (%)	...	0.38
Tests on protein component		
Casein (%)	...	Less than 0.1
Lactalbumin + lactoglobulin (%)		14.4
pH	5.57	5.63
Cal./100 gm	355	374

Whey used in these experiments, both the dialyzed and the undialyzed, was from the same batch of cheddar cheese whey which was processed very carefully to avoid denaturization. The whey remained completely water soluble even after drying. The dialysis treatment was relatively brief, by no means exhaustive. Hence, the results reported here for the dialyzed whey may not necessarily be the most favorable ones.

The changes brought about by dialysis may be seen by the comparative analysis given in table 2.

The most significant difference between the 2 analyses is in the amount of soluble ash. The soluble ash is reduced out of proportion to the total ash because a certain fraction of the calcium and phosphorus is an integral part of the protein complex and cannot be removed by dialysis.

In the final test, a diet containing dialyzed whey was compared with an identical diet without whey, the only variation in composition being in the ratio of lactalbumin and casein. In order to have the same mineral content of 3.44% in each case, 0.78% minerals (Osborne-Mendel mixture) were added to the control diet.

Test no. 3

This was a comparison of a diet based on milk proteins only (Control) with a high lactalbumin diet containing milk plus dialyzed whey proteins (Test) (columns 6 and 7 in table 1).

The animals fed on the test diet ate 11.4% in excess, and gained 26.3% more than those on milk proteins; in all they showed a 14.5% greater gain in body weight per gm food intake than the rats on the control diet.

During the experimental period, animals in both groups were normal and healthy in appearance. Diarrhea was prevalent among the rats receiving the test diet during the first week and again during the sixth week.

CONCLUSIONS

In normal (undialyzed) whey the biological value of the lactalbumin is masked so that it appears to be less than that of casein. This may explain why whey is not now being used

in infant feeding formulae in spite of the recognized food value and desirability of its 2 chief constituents: lactose and lactalbumin, and the availability of whey at low cost. In dialyzed whey the true biological value of the lactalbumin becomes evident, demonstrating its superiority over casein by a considerable margin.

Mineral constituents of the whey are responsible for its low nutritional value, which is associated with lessened appetite, a tendency for diarrhea and an impaired ability to digest carbohydrates, as shown by the higher galactose excretions. The reason for, and the mechanism by which these changes are brought about, are not apparent from these tests.

Rats fed on the dialyzed whey formula showed greater appetite than the rats fed on a milk formula only.

Dialysis is capable of converting whey, a dairy by-product, into a highly valuable ingredient for infant formulae. Whether the degree of dialysis arbitrarily chosen here results in the best possible product for this or other purposes, remains to be determined by future experiments.

SUMMARY

1. Partial substitution of whey proteins for casein, in a formula resembling human milk, detrimentally affects the growth of rats.

2. Removal of a considerable portion of minerals from whey by means of dialysis results in a more favorable growth response. On a diet containing dialyzed whey, the rats grow 12.8% better per gm of food intake than on the same diet containing undialyzed whey.

3. If the minerals removed from the whey by dialysis are reintroduced to the dialyzed whey after ashing, the rats' growth again is impaired. This proves that 1 or more of the inorganic constituents, rather than an organic compound, affects detrimentally the nutritional value of whey.

4. On a diet in which 53% of casein is replaced by dialyzed whey lactalbumin, rats show 14.5% greater efficiency in conversion of food to body tissue than rats fed on a corresponding

formula containing milk proteins only. This experimental result is in agreement with previous work whereas the results with undialyzed whey seemed to dispute the well established biological superiority of lactalbumin over casein.

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NITROGEN RETENTION STUDIES ON RATS, DOGS AND MAN; THE EFFECT OF ADDING METHIONINE TO AN ENZYMIC CASEIN HYDROLYSATE

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TWO FIGURES

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The literature is replete with evidence that casein contains but little cystine and that its nutritive value can be improved by the addition of small quantities of this amino acid. The early findings by Osborne and Mendel ('15) that casein would not support as rapid growth in rats at a 9% level as lactalbumin is the basis of the oft-cited nutritional superiority of mother's milk to cow's milk. Marriott and Jeans ('41) state: "Human milk contains less protein than cow's milk, but of the total protein present, almost 60% is lactalbumin, as compared with about 15% in cow's milk. Though the proteins of both these milks are of high nutritional value, that of cow's milk is of less value than that of human milk, possibly as much as 20% less."

[1] That portion of the study conducted at Washington University School of Medicine was aided by a grant from the Commonwealth Fund.

Some divergence from this view is expressed by Gordon, Levine, Wheatley and Marples ('37) who found that premature babies fed high but equal levels of protein from cow's and mother's milks retained equal amounts of nitrogen. Harrison ('36) fed a low nitrogen intake as cow's milk to normal infants and reported adequate nitrogen retention.

With the advent of parenteral alimentation and the use of casein as one of the principal sources of protein for hydrolysis, it becomes particularly necessary to know to what extent casein is deficient for human nutrition. The physical problems of parenteral alimentation properly emphasize the importance of choosing that nitrogen source most efficient for retention.

The results of animal studies on the addition of cystine to casein have never been confirmed in human beings. The only report on the nutritional value of added cystine for man is that of Pittman ('32) who added 2% of cystine to navy beans and found a slightly improved nitrogen retention. In the present study, rats, dogs, and human subjects have been employed in determining the value of adding cystine or methionine to a casein hydrolysate.

EXPERIMENTAL

Studies on dogs

In order to simulate the clinical conditions under which the hydrolysates would be used parenterally, 2 dogs were depleted in weight and protein stores by long feeding of the Weech-Goettsch low-protein diet ('38) to which was added a daily supplement of vitamins and a small amount of commercial dog food to supply in total 0.04 gm of nitrogen and 50 cal. per kilo. At the end of the depletion period of 12 weeks, the first dog had lost 128 gm of nitrogen, 1.25 kg in weight, and had a total plasma protein of 5.6 and albumin 3.8 gm % and a hematocrit reading of 48. Toward the end of the subsequent experimental periods a portion of the food was not eaten and the caloric intake was inadequate (see table 1).

TABLE 1

The effect of adding individual amino acids to casein hydrolysate given intravenously.
Dog 68; mongrel male.

PERIOD (7 DAYS)	SUPPLEMENT [1]	DAILY CALORIC INTAKE PER KG	DAILY N INTAKE GM/DAY		TOTAL NITROGEN EXCRETION IN GM		AVERAGE DAILY N BALANCE GM	WEIGHT KG	PLASMA PROTEINS [3]		
			Food	Intravenous	Urine	Feces			Total gm %	Albumin gm %	Hematocrit
11	None	50.0	0.82	...	10.71	3.96	—1.28	20.4	..	..	..
12	None	50.2	0.82	...	10.26	4.38	—1.27	20.5	5.7	3.8	58
13	Hydrolysate	51.4	0.82	1.16	12.03	3.85	—0.30	20.4	6.5	3.1	60
14	H + 5% VUK [2]	51.6	0.83	1.16	11.47	4.59	—0.31	20.9	6.3	3.2	56
15	H + 5% dl-threonine	51.0	0.83	1.16	11.42	4.32	—0.26	20.8	5.7	2.5	58
16	H + 5% dl-valine	51.7	0.84	1.16	10.37	4.66	—0.19	21.1	5.2	2.5	50
17	H + 5% dl-leucine	51.1	0.84	1.16	10.87	3.71	—0.09	21.1	5.7	2.6	53
18	H + 5% dl-isoleucine	51.4	0.85	1.16	10.62	4.61	—0.17	21.3	5.5	2.6	51
19	H + 5% l(+) lysine HCl	51.5	0.86	1.16	10.99	4.03	—0.13	21.4	5.3	2.7	53
20	H + 5% l(—) tryptophane	51.5	0.87	1.16	11.70	4.62	—0.31	21.7	5.5	2.5	52
21	H + 5% dl-phenylalanine	51.2	0.86	1.16	11.69	4.08	—0.24	21.5	..	2.5	54
22	H + 5% dl-methionine	48.8	0.84	1.16	7.43	4.69	+0.27	21.9	5.4	2.6	54
23	H + 5% l(+) histidine HCl	35.1	0.61	1.16	9.03	3.27	+0.01	22.0	5.9	2.9	52
24	H + 5% l(+) arginine HCl	33.0	0.58	1.16	10.30	4.05	—0.31	21.4	6.9	3.3	65
25	Hydrolysate	31.0	0.55	1.16	10.51	4.11	—0.38	21.4	5.8	2.9	56
26	H + 5% dl-methionine	43.7	0.74	1.16	7.19	3.25	+0.40	21.1	6.1	3.0	55
27	Hydrolysate	36.3	0.63	1.16	9.87	4.58	—0.28	21.2	5.5	2.9	56
28	H + 5% dl-leucine	36.7	0.69	1.16	10.63	3.56	—0.19	21.4	5.6	2.7	57
29	H + 5% dl-valine	34.3	0.59	1.16	9.90	3.57	—0.18	21.2	5.4	2.5	57
30	Commercial dog food	26.9	1.58	...	9.96	4.82	—0.53	21.1	5.5	2.6	54
31	H + 5% l(+) lysine HCl	32.2	0.54	1.16	10.23	3.48	—0.26	20.9	5.7	2.6	57
32	H + 5% l(+) histidine HCl	30.3	0.51	1.16	9.68	3.13	—0.15	20.5	5.7	2.8	56

[1] The supplement replaced 5% of the hydrolysate nitrogen.

[2] Mixture of essential amino acids devised by Madden et al., J. Exp. Med., *82:* 77.

[3] The values obtained at the end of the indicated period are given.

A 10% solution of the casein hydrolysate [2] sterilized by Seitz filtration, in an amount calculated to equal the daily nitrogen loss during the last few depletion periods was infused intraveneously at a constant rate of 1.33 mg N/kg/min. once daily for 7 days. During subsequent periods 5% of the hydrolysate nitrogen was substituted by an equal amount of nitrogen from 1 of the essential amino acids. Each test mixture was given for 7 days. The tabulated results are given in table 1. The last 2 weeks of depletion are included in the table for comparison.

The intravenous injection of 1.16 gm nitrogen as the hydrolysate did not lead to a positive balance, but spared about 0.97 gm of body nitrogen (Period 13). The substitution of a small amount of all 10 mixed essential amino acids did not improve retention (Period 14). Single amino acids seemed to exert a slight improvement but the only important change was caused by methionine (Periods 22 and 26). These 2 periods with added methionine gave a net nitrogen *retention* of 0.33 gm daily, which is to be compared with the 3 periods on the hydrolysate alone (Periods 13, 25, 27), which gave an average daily *loss* of 0.32 gm nitrogen. The nitrogen balance when valine, leucine, lysine and histidine were added was suggestive of improvement (Periods 16, 17, 19 and 23), but this was not confirmed on repetition (Periods 28, 29, 31 and 32).

The validity of the last few periods in this series is in question since the dog progressively refused to eat, with consequent drop in caloric intake. In Period 30, the 1.16 gm of nitrogen that had been given intravenously were fed in the form of commercial dog food, but a large amount of the carrot diet was refused.

The results with methionine were extended and confirmed in a second dog kept on the protein-poor diet for 11 weeks (nitrogen intake 0.04 gm per kg; caloric intake, 50 per kg), during which time he lost 6 kg in weight. Nitrogen loss was deter-

[2] All casein hydrolysate used in this study was prepared by pancreatic digestion in a manner previously described (Mueller, Kemmerer, Cox and Barnes, '40). The preparation is known under the trade name of Amigen.

TABLE 2

Effect of supplemental methionine on nitrogen retention when an enzymic casein hydrolysate is given orally, intravenously and subcutaneously.

Dog 103; male pointer.

PERIOD (7 DAYS)	SUPPLEMENT	DAILY CALORIC INTAKE PER KG	NITROGEN INTAKE GM/DAY		NITROGEN EXCRETION GM		AVERAGE DAILY N BALANCE GM	WEIGHT KG	PLASMA PROTEINS		
			Food	Supplement	Urine	Feces			Total gm %	Albumin gm %	Hematocrit
10	None	50.0	0.61	...	12.90	5.56	— 2.03	15.2	5.6	1.6	45
11	None	50.3	0.60	...	11.63	5.32	— 1.85	14.9	5.3	1.5	41
12	Hydrolysate, i.v.	54.1	0.60	1.81	14.93	3.97	— 0.29	14.9	5.7	1.6	42
13	Hydrolysate, i.v.	53.7	0.59	1.90	14.37	4.87	— 0.26	14.9	5.3	1.8	39
14	Hydrolysate + Methionine, i.v.	53.4	0.60	1.90	12.46	3.51	+ 0.22	15.0	5.9	1.9	37
15	Hydrolysate, i.v.	53.7	0.60	1.90	14.67	4.30	— 0.21	15.1	5.7	1.7	37
16	Hydrolysate + Methionine, i.v.	53.6	0.59	1.90	11.07	4.29	+ 0.29	14.7	5.7	1.8	36
17	Hydrolysate, i.v.	54.2	0.60	1.90	11.92	4.27	+ 0.19	15.1	5.5	1.6	37
18	Hydrolysate, i.v.	54.7	0.60	1.90	13.53	3.58	+ 0.06	14.9	5.8	2.0	41
19	Hydrolysate + Methionine, i.v.	53.5	0.59	1.90	9.62	3.62	+ 0.60	14.7	6.0	2.2	42
20	Hydrolysate, oral	54.3	0.60	1.90	11.02	3.44	+ 0.44	14.7	5.6	2.1	44
21	Hydrolysate + Methionine, oral	54.2	0.60	1.90	6.98	3.03	+ 0.93	15.0	5.7	2.8	44
22	Hydrolysate, oral	53.3	0.60	1.90	8.67	3.57	+ 0.75	15.1	5.3	2.3	44
23	Hydrolysate, oral	54.9	0.61	1.90	12.34	4.18	+ 0.15	15.0	5.5	2.3	45
24	Hydrolysate, oral	54.9	0.60	1.90	13.95	4.04	— 0.07	14.9	5.7	2.3	48
25	Hydrolysate, s.c.	53.5	0.59	1.90	13.96	3.64	— 0.03	14.7	5.4	2.4	..
26	Hydrolysate + Methionine, s.c.	53.9	0.59	1.90	8.18	2.42	+ 0.98	14.7	5.9	2.4	44
27	Hydrolysate, i.v.	54.6	0.60	1.90	10.16	4.08	+ 0.47	15.1	5.8	2.7	46

mined for the last 5 weeks of depletion and averaged 1.90 gm daily. Intravenous supplementation with this amount of hydrolysate nitrogen (Periods 12 and 13) was followed by certain periods in which 5% of the hydrolysate nitrogen was replaced with an equal amount of methionine nitrogen. The data are given in table 2. It will be noticed that 1.90 gm hydrolysate nitrogen spared 1.68 gm of body nitrogen (Periods 10, 11 and 13), but the substitution of methionine resulted in actual nitrogen retention (Period 14). The improved nitrogen retention with the methionine supplement persists whether the material is given intravenously (compare Periods 13, 14 and 15), orally (compare Periods 20, 21, 22, 23 and 24) or subcutaneously (Periods 25 and 26). The beneficial effect of

TABLE 3

Comparison of nitrogen balance after administering casein hydrolysate by different routes.

DOG 103	GM NITROGEN PER DAY	
Nitrogen source	Casein Hydrolysate	Casein Hydrolysate + Methionine
Intravenous (Periods 13, 14, 16, 19)	— 0.26	+ 0.37
Subcutaneous (Periods 25, 26)	— 0.03	+ 0.98
Oral (Periods 24, 21)	— 0.07	+ 0.93

methionine on nitrogen retention persists for at least 2 weeks following its withdrawal. This is best shown in Period 21 when the oral administration of the hydrolysate with methionine resulted in a retention of 0.93 gm daily, and when the methionine was omitted in 3 subsequent periods, the retention dropped progressively, + 0.75, + 0.15, — 0.07 gm daily.

The data permit a rough comparison of different routes of administration. As shown in table 3, the subcutaneous and oral routes give definitely higher retentions than the intravenous. When the hydrolysate was given without supplementation, intravenous infusion gave a nitrogen balance of — 0.26; subcutaneous + 0.98 (Period 26); and oral + 0.93 (Period 21) gm daily. This difference may be due in part to a

greater renal excretion of amino acids or peptides following intravenous infusion (Cox and Mueller, '46).

Studies on rats

Two hydrolysates of casein prepared in an identical manner except that 1 was digested with tryptic enzyme from pancreas (Mueller, Kemmerer, Cox and Barnes, '40) and the other with tryptic enzyme from the pyloric caeca of fish (Bondouy, 1899), were each fed to 10 young rats at a level of 1.2% nitrogen (equivalent to 7.5% protein). Additional groups were fed similarly except that 2½% [3] of hydrolysate nitrogen was replaced by an equal amount of cystine and of methionine nitrogen, so that the total nitrogen intake (1.2%) remained constant. Young albino rats weighing between 35 and 45 gm, and distributed as to sex and litter, were placed at weaning on each of the 6 experimental diets. The rats were housed in individual wire-bottomed cages and fed tap water and the diet ad libitum. The basal diet consisted of lard 9.0, salt mixture 4.0, cod liver oil 2.0, wheat germ oil 1.0, brewers' yeast 2.0, thiamine hydrochloride 0.0006, and riboflavin 0.0002%, and enough hydrolysate added to supply 1.2% nitrogen and dextrin sufficient to make 100 gm.

The average weight gains are given in table 4. As expected from the early work of Osborne and Mendel ('15) and Womack, Kemmerer and Rose ('37), both supplemented diets resulted in increased weight gain. Methionine consistently gave greater gains than cystine. In order to determine that the gain in weight was not due to water or fat retention, the rats were killed at 8 weeks, the gastrointestinal tract removed with as little loss of blood as possible, and the individual rats placed in pint jars and autoclaved under steam pressure. They were ground and analyzed individually for total

[3] This level was chosen after preliminary experiments in which 1.25, 2.5, 5.0, 7.5 and 10% of methionine nitrogen had been substituted for an equal amount of hydrolysate nitrogen. Growth on the 2 lowest levels was equal, and greater than on the other 3.

TABLE 4

Average gain in weight and per gram nitrogen ingested of 10 rats fed 1.2% nitrogen for 8 weeks as an enzymic hydrolysate of casein with supplements of cystine and methionine (2½% of the hydrolysate nitrogen).

HYDROLYSATE EMPLOYED	AVERAGE GAIN IN WEIGHT		GAIN PER GM N INGESTED	
	A	P	A	P
	gm	*gm*	*gm*	*gm*
Hydrolysate without supplement	112	91	15.7	14.6
Hydrolysate with cystine	131	115	18.9	17.8
Hydrolysate with methionine	154	129	20.6	19.0
Gain due to cystine	19	24	3.2	3.2
Gain due to methionine	42	38	4.9	4.4

TABLE 5

Average composition of rats and calculated composition of the gain as total solids, protein, ash and fat.

	% COMPOSITION				COMPOSITION OF THE GAIN IN GM PER AVERAGE RAT			
Substance fed	Solids	Nitrogen	Ash	Lipids	Solids	N × 6.25	Ash	Lipids
Hydrolysate A	40.28	3.23	3.85	16.77	45.1	22.6	4.31	18.8
Hydrolysate + Cystine	39.09	3.36	3.98	14.50	51.2	27.5	5.21	19.0
Hydrolysate + Methionine	39.10	3.31	3.76	15.19	60.2	31.9	5.79	23.4
Hydrolysate P	39.08	3.34	4.35	14.34	35.6	19.0	3.96	13.1
Hydrolysate + Cystine	39.34	3.28	4.05	15.08	45.2	23.6	4.66	17.3
Hydrolysate + Methionine	40.63	3.28	3.74	16.36	52.4	26.4	4.82	21.1

solids, ash, protein and fat, using standard analytic methods.[4] The results are given in table 5. There is no significant difference in the per cent total solids, nitrogen, ash or lipids when the hydrolysate group is compared with the groups receiving additional sulfur-containing amino acids. The actual

[4] We wish to express our appreciation to Mr. Charles Wesselman for these analyses.

composition of the gain shows that there was an absolute increase in body protein, ash, solids and fat, which paralleled the increase in total weight. If the weight of the rat is plotted against the quantitative gain in each of these 4 categories, the values fall along a straight line, indicating that the gain in weight of the methionine and cystine rats was a real gain and not due to retention of water or to the deposition of excessive fat.

Observations on human beings

Patients. Four groups of human subjects have been used in studying the effect of added methionine. The first group consisted of 3 patients with pyloric obstruction who were being prepared for operation. For the period of this experiment they took no food of any kind by mouth and all gastric secretions were constantly drained by tube. The patients were all emaciated, having lost from 20 to 40 pounds in body weight and were obviously in need of protein. As the sole source of food, 2 liters of Amigen 5% in 5% Dextrose Solution were given each patient daily for 5 days and the amount of urinary nitrogen and that lost by gastric lavage determined daily. No fecal samples were excreted since no food was ingested. During a second consecutive 5-day period, 3.2 gm of methionine replaced an equal amount of hydrolysate, the same amount of total nitrogen and calories being injected daily as in the 5 previous days. As shown in table 6, 1 of the 3 subjects was in nitrogen balance at this level of nitrogen and caloric alimentation while the other 2 were in slightly negative balance. The addition of methionine to the solution of the hydrolysate during the second 5-day period actually resulted in a worsened balance in 2 of the patients and an improvement in the other. From this it was clear that the methionine had had no beneficial effect on nitrogen retention.

Normal infants. We were somewhat puzzled at this negative finding for methionine and thought that it might possibly have been due to an inadequate caloric intake, or to some abnormality of metabolism related to the existing gastrointestinal

disease. With the idea that the beneficial effects of added methionine, if any, might be more clearly demonstrated in the growing organism, nitrogen balance experiments were performed on 2 normal, white, full-term male infants. They were maintained on a standard metabolism frame adapted for

TABLE 6

Nitrogen balance of 3 male patients with pyloric obstruction being prepared for gastric resection. Two liters of Amigen 5% in 5% dextrose solution intravenously with and without added methionine (a total of 12.7 gm nitrogen) were given each patient daily.

Day	PATIENT F: 63 years; wt. 45 kg			PATIENT H: 66 years; wt. 51 kg			PATIENT M: 62 years; wt. 67 kg		
	Urinary N	Gastric contents N	N balance	Urinary N	Gastric contents N	N balance	Urinary N	Gastric contents N	N balance
	gm	gm	gm	gm	gm	gm	gm	gm	gm
	Hydrolysate infusion without supplement								
1	8.20	0.68	+ 3.8	11.48	0.44	+ 0.8	13.65	0.50	— 1.5
2	8.27	1.63	+ 2.8	14.28	0.12	— 1.7	13.89	0.52	— 1.7
3	9.80	1.29	+ 1.6	11.48	0.19	+ 1.0	16.42	0.29	— 4.0
4	8.93	1.15	+ 2.6	15.43	0.12	— 2.9	15.39	0.26	— 3.0
5	9.15	1.16	+ 2.4	11.96	0.19	+ 0.5	13.94	0.32	— 1.6
Average			+ 2.6			— 0.5			— 2.4
	With methionine; 3.2 gm daily replacing an equal amount of hydrolysate								
6	12.31	1.15	— 0.8	15.07	1.03	— 3.4	12.50	0.69	— 0.5
7	9.98	0.98	+ 1.6	14.08	0.09	— 1.5	13.06	inc.	— 0.4
8	10.62	0.90	+ 1.2	14.14	inc.	— 1.4	15.03	0.15	— 2.5
9	11.03	0.86	+ 0.8	15.45	0.06	— 2.8	14.69	0.11	— 2.1
10	inc.	inc.	..	13.08	0.08	— 0.5	14.01	0.07	— 1.4
Average			+ 0.7			— 1.9			— 1.4

the collection of urine and feces. The diet periods began on each Saturday morning and during the first 2 days the urine and feces were not collected in order to allow adjustments to the diet and rest from continued restraint. The infants were placed on metabolism frames at the end of these 2 days and daily collections continued for 5 days. The diet was composed

of casein hydrolysate, 3.5 gm; Laboratory Product no. 217,[5] 18 gm; dried brewers' yeast, 1 gm dissolved in sufficient water to make 100 ml. One cubic centimeter of the solution supplied 1 calorie.

The diet was given in 5 feedings daily at 100 cal. per kilo. body weight and was supplemented with 50 mg of ascorbic acid and 15 drops of Oleum Percomorphum. The daily intake was calculated from the food consumption record, and from the

TABLE 7

Nitrogen balance in infants, effect of adding methionine to a casein hydrolysate.

	PATIENT P. D., 7381 GM WEIGHT		PATIENT D. R., 7073 GM WEIGHT	
Nitrogen source	Hydrolysate	H + Methionine	Hydrolysate	H + Methionine
Period, 5 days each	1	2	1	2
Total Nitrogen intake, gm	5.03	5.20	4.70	4.84
Nitrogen absorption, mg/kg/day	585	603	639	671
Nitrogen retention, mg/kg/day	182	182	309	297
Nitrogen retention, gm/day	1.40	1.45	2.19	2.14
Gain in weight, gm/day	36.0	32.4	9.7	28.7

analysis of a composite sample of the 7 daily formulas. During the second period 3% of methionine was added to the casein hydrolysate and all other quantities maintained constant.

Nitrogen absorption was calculated by subtracting fecal nitrogen from the intake; and retention by subtracting the urinary nitrogen from that absorbed. It is evident from table 7 that the net retention, expressed in grams per day, was identical in the supplemented and unsupplemented periods.

[5] Consisting of Dextri-Maltose no. 2, 54.6; milk fat, 23.9; arrowroot starch, 13.0, calcium gluconate, 4.4; KH_2PO_4, 1.8; $Ca(OH)_2$, 1.1; K_2HPO_4, 0.6; KCl, 0.5; Mg O, 0.1 and $FeSO_4$, 0.02 gm.

The addition of methionine did not improve nitrogen retention. This second failure of methionine to improve nitrogen retention even in growing babies was most surprising. However, the cystine deficiency of casein is only demonstrable in the rat at low levels of protein intake (Osborne and Mendel, '15); and these balance studies on babies were made at a nitrogen intake equivalent to 4 to 5 gm of protein per kg body weight. This intake level may possibly have been sufficiently high to mask any inadequacy of the casein hydrolysate.

Normal adults at a maintenance level of nitrogen. In view of these 2 negative results for added methionine in the human subjects, we felt that further studies with minimal intakes might give results more in accord with those found in rats and dogs. Two groups of adult volunteers,[6] all laboratory workers, collaborated in the studies. One group of 6 subjects was studied at a maintenance level, and the other group of 4 subjects was on a sub-maintenance level. All subjects were completely normal on physical examination, including the usual blood and urine analyses. In the maintenance study 3 men and 3 women were placed for 7 days on a regulated diet of natural foods supplying 40 cal. and 0.11 gm of nitrogen per kg daily. The protein-low diet of Mueller, Fickas and Cox ('43) was then substituted but the nitrogen intake level was kept constant at 0.11 gm per kg by the addition of casein hydrolysate. The low protein diet supplied 0.01 gm of nitrogen per kg or 9% of the total nitrogen intake. Daily urinary and fecal nitrogen determinations were made. It is evident from figure 1 that the hydrolysate was as effective in maintaining nitrogen balance (Periods 3 and 4) as regular food protein (Period 2).

The hydrolysate was then removed from the diet for a period of 4 days, with marked nitrogen loss (Period 5). When restored at the same level as previously, there was nitrogen

[6] These volunteers were: Misses Edna Fisher, Kathryn Drone, Theodora Block, Messrs. William Koch, Edward Bonham, Donald Schneider, K. S. Kemmerer, R. W. Barton, A. J. Mueller and W. M. Cox, Jr. We want to express our appreciation of their help.

retention (Period 6). During the last 4-day period, 2½% of the hydrolysate nitrogen was replaced by an equal amount of cystine nitrogen (Period 7) but without any increase in nitrogen retention. This finding is again in contradistinction to the animal findings. In trying to rationalize why there should be such a marked difference in the effect of added methionine

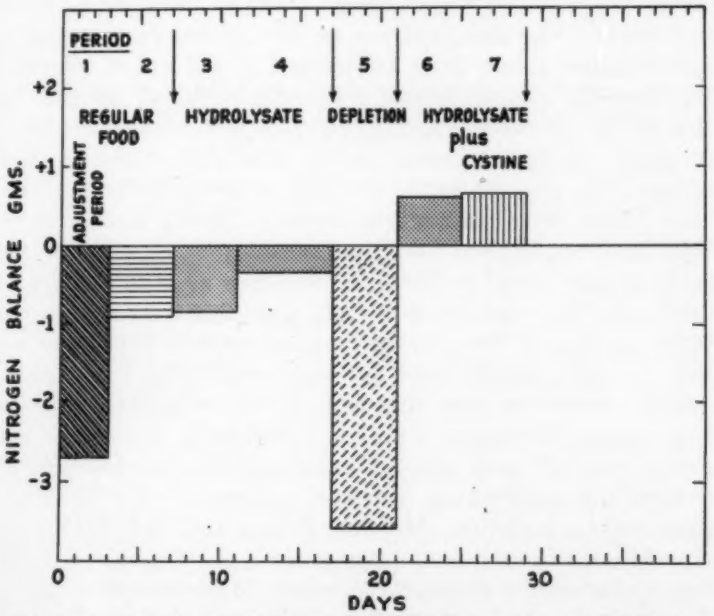

Fig. 1 Average nitrogen balances of 6 adult human subjects maintained on a diet supplying 0.11 gm of nitrogen and 40 cal. per kg body weight daily. During the periods when the enzymic casein hydrolysate was given, the protein-low diet of Mueller, Fickas and Cox ('43) was given. Daily balances were determined and averaged as 3, 4 or 6-day periods.

in 2 species, it was evident that (1) the dogs were protein-depleted whereas the humans were merely on a maintenance diet without any significant depletion and (2) the nitrogen intake of the human subjects was the same per kg body weight, whereas the intake of the dogs was adjusted to their endogenous loss.

Normal adults depleted of protein. As a final test for the value of added methionine in man, it was decided to deplete our 4 human volunteers of their protein stores in a manner similar to that employed with the dogs and then to supplement the diet by that amount of nitrogen which each was losing daily. Four normal men ingested the protein-low diet previously described (Mueller, Fickas and Cox, '43) for a period of 3 weeks. Nitrogen analyses on the foodstuffs were made and the actual intake level established at 0.01 gm of nitrogen per kg. The caloric intake was maintained at 30 per kg throughout the entire experiment. This is 1.4 times the average basal caloric requirement when calculated on the basis of surface area (height-weight formula of DuBois and DuBois, '16). Other investigators, particularly Martin and Robison ('22) have emphasized the difficulty of ingesting protein-free foods for protracted periods. While adjustment to the diet of starch-milkfat crackers, milk fat, vegetable oils, sugar and small amounts of low-nitrogen vegetables and fruits, as lettuce, cabbage, peaches and apricots was not easy, the principal objection was that of monotony. All subjects continued their usual laboratory and home-gardening activities, but about 1 day in 5 each was depressed and felt that he couldn't continue the undertaking. Our early observations of lassitude after 4 days depletion (Mueller, Fickas and Cox, '43) were probably due to such temporary periods of adjustment to the diet, which usually disappeared within 24 hours with a return of a normal reaction towards activity and duties. Urinary and fecal nitrogen was determined daily, and the nitrogen in coffee was counted as part of the total nitrogen intake.

As noted above, all subjects were proved normal by the usual clinical and chemical examinations and the measurements of most interest for the purpose of this study — weight, total serum protein and serum albumin, and hematocrit — are given in table 8. During depletion, similar measurements were made at weekly intervals and at the end of the experimental period (table 8). It will be seen from these data that the weight loss was between 3.2 and 5.2 kg. The nitrogen loss during the

whole experiment multiplied by the customary 30 to convert it to a weight equivalent gave an indicated weight loss of from 2.2 to 3.2 kg in the 4 subjects. This calculated weight loss is not greatly different from the actual weight loss, indicating that the caloric intake of 30 per kg was adequate, although it should be noted that subject (A), performing the most work, lost the greatest weight. Other investigators (Martin and

TABLE 8

Weight, serum protein, serum albumin and hematocrit values during protein depletion of 4 subjects for 21 days, and after 10 days of minimal hydrolysate supplementation.

SUBJECT	A	B	C	D
	kg	*kg*	*kg*	*kg*
Weight, initial	91.2	72.1	84.7	82.1
1 wk. depletion, loss	—1.8	—0.9	—0.2	—1.8
2 wks. depletion, loss	—0.7	—2.4	—1.6	—0.7
3 wks. depletion, loss	—2.2	—0.2	—0.3	—0.8
End of supplementation	—0.5	—0.2	—1.2	+0.1
Total weight loss	—5.2	—3.7	—3.3	—3.2
Total nitrogen loss, gm	106.7	89.4	71.4	89.3
N. loss × 30, kg	3.2	2.7	2.2	2.7
	gm %	*gm %*	*gm %*	*gm %*
Serum albumin, initial	4.66	4.91	4.91	4.67
1 wk. depletion	4.35	4.60	4.62	4.32
2 wks. depletion	4.31	4.81	4.17	4.10
3 wks. depletion	4.18	4.37	4.39	4.23
Change during depletion	—.48	—.54	—.52	—.44
End of supplementation	3.99	4.50	4.43	4.37
	gm %	*gm %*	*gm %*	*gm %*
Serum protein, initial	6.87	7.09	7.30	7.43
1 wk. depletion	7.06	6.72	6.84	6.46
2 wks. depletion	6.54	7.05	6.26	6.10
3 wks. depletion	6.25	6.56	6.80	6.19
Change during depletion	—.62	—.53	—.50	—1.24
End of supplementation	6.39	6.79	6.59	6.46
	% c.v.	*% c.v.*	*% c.v.*	*% c.v.*
Hematocrit, initial	45.1	48.5	49.9	46.3
1 wk. depletion	46.0	47.3	51.4	48.0
2 wks. depletion	47.2	47.0	50.3	44.4
3 wks. depletion	49.5	43.2	55.9	44.1
End of supplementation	47.0	40.6	51.8	42.6

Robison, '22) have employed higher caloric intakes, but a large part of their difficulty with the ingestion of the diets is probably due to this high food intake.

The total serum protein and serum albumin values declined progressively during depletion, in a manner and degree comparable to that previously observed in dogs (Cox and Mueller, '44). There was no significant regeneration of protein during supplementation, since the level of nitrogen given was inadequate to attain nitrogen balance. There was but little change in the hematocrit values during the experiment, so that the fall in percentage albumin may be considered as valid.

TABLE 9

Summarized data on 4 subjects for the last 6 days of a 21-day protein depletion period followed by two 4-day periods supplemented with casein hydrolysate with and without added methionine.

SUBJECT	A	B	C	D
Food N, gm/day, last 6 days	0.91	0.72	0.85	0.82
Urinary N, gm/day, last 6 days	3.44	2.88	2.33	2.68
Fecal N, gm/day, last 6 days	1.03	0.40	0.79	0.70
Net nitrogen loss during last 6 days of depletion	—3.56	—2.56	—2.27	—2.56
Nitrogen balance during hydrolysate supplementation	—0.97	—0.96	—0.76	—1.36
Nitrogen balance during hydrolysate and methionine supplementation	—0.99	—1.06	—0.91	—1.29

At the end of 21 days depletion, hydrolysate was added to the diet. The amount given was equal to the average amount of nitrogen lost by each subject during the last 6 days of the 21-day period and was administered for the next 4 consecutive days as the casein hydrolysate. As shown in table 9 (line 4), the net nitrogen loss was 3.56, 2.56, 2.27 and 2.56 gm daily for the 4 subjects. These values are in very close agreement with the endogenous loss reported by others under similar protein depletion. For example, Martin and Robison ('22) report an endogenous loss of 3.17 and 2.89 gm nitrogen per

day. The average amount of hydrolysate required to furnish the required amount of nitrogen was only 22.3 gm daily. As shown in line 5 (table 9), balance was not achieved although the nitrogen loss was considerably reduced by the supplement. The average loss of — 2.74 gm was reduced to — 1.01 gm daily. A second period followed in which 2½% of the hydrolysate nitrogen was substituted by an equal amount of nitrogen

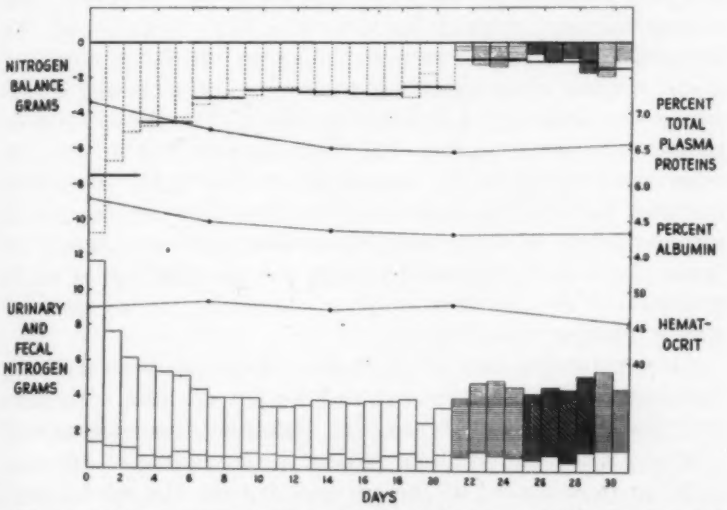

Fig. 2 Nitrogen balance, urinary and fecal nitrogen and blood values of 4 human adult males receiving a protein-low diet (0.01 gm N per kg per day) and 30 cal. per kg body weight for a period of 21 days. Supplementation with an enzymic casein hydrolysate for 4 days (horizontal lines, 22 to 25th day) and with the same hydrolysate and a methionine supplement (diagonal lines, 26 to 29th day) for 4 days followed depletion. The values for each day are given and the heavy lines indicate the average nitrogen balance for 3- or for 4-day periods.

as methionine. The average weighed amount of methionine was 0.763 gm daily for each of the 4 subjects. As shown in the last line of table 9, and in figure 2, the added methionine led to no more reduction in nitrogen loss than the hydrolysate alone, since the average nitrogen balance for the 4 subjects was — 1.06 gm daily, as compared with — 1.01 for the hydrolysate period immediately preceding.

This finding demonstrated to us that the addition of methionine to a casein hydrolysate did not increase the efficiency of nitrogen retention in humans.

DISCUSSION

A comparison of the nitrogen retention of a casein hydrolysate with and without added methionine in rats, dogs and man has clearly shown a striking species difference. The addition of methionine increased the rate of growth in rats and the magnitude of nitrogen retention in dogs. In man, however, it was without effect on nitrogen retention even though observations were made on 4 different groups: (1) depleted surgical patients fed intravenously (2) normal adults fed at a maintenance nitrogen level (3) normal adults who had been protein depleted for 3 weeks and were then fed only an amount of nitrogen equal to their endogenous loss, and (4) normal infants. In no one of these 4 groups did the addition of extra amounts of the essential sulphur-containing amino acid increase nitrogen retention.

An explanation for this difference does not seem difficult, based on the fact that the rat and dog are covered with hair, and that man is not. Since hair contains large amounts of cystine (Block and Bolling, '45) it is reasonable to suppose that the requirement of the rat and dog for this amino acid (or methionine) is considerably greater than that of man.

Whether this explanation is valid or not, the implications of the finding are large. We employed an hydrolysate of casein but it seems reasonable to interpret the data as though casein itself were employed, inasmuch as the growth of rats receiving the hydrolysate was equal to that of those receiving casein (Mueller, Kemmerer, Cox and Barnes, '40). Moreover, supplementation of the hydrolysate with cystine and methionine gave the same improvement in growth as when casein (unpublished studies) was similarly supplemented.

The finding of Osborne and Mendel ('15) that casein was deficient in cystine was based on rat observation. It was promptly (and at that time, perhaps properly) used in ex-

plaining the contemporary observation that infants failed to develop as well on artificial feedings as on human breast milk (Holt, '16). Thus, it was argued that since the protein of cow's milk consists preponderantly of casein with a low cystine content, whereas breast milk contains principally lactalbumin with a high content of sulfur-containing amino acids, it is logical to attribute the inferior results from cow's milk feedings to this fact. With improvement in the bacteriological quality of cow's milk, the supposed nutritional differences between casein and lactalbumin became much less necessary or evident. Gordon, Levine, Wheatley and Marples ('37) studied infants fed similar amounts of nitrogen as cow's milk and as breast milk and could find no significant difference in their nitrogen retention. Harrison ('36) reported that when the intake of cow's milk protein was quantitatively reduced to that ingested in breast milk, good nitrogen retention was observed. These earlier observations support the present finding.

On the basis of the work of Osborne and Mendel ('15) it would have been logical to add cystine to cow's milk to increase its nutritive value. To our knowledge, this experiment was not done. Our findings indicate that neither the addition of cystine or methionine should have increased the nutritive value of the milk for babies, even though it might have done so for the rat. That there is no difference in the nutritive value of casein and lactalbumin for man based on a difference in sulfur-containing amino acid content is clear from the present findings, but to establish the relative nutritive value of these 2 proteins, casein and lactalbumin, *for man,* it will be necessary to compare carefully their growth-promoting qualities and their effectiveness for nitrogen retention *in man.* Experimental work with rats will not suffice. The assumed nutritive difference between casein and lactalbumin has been established *for the rat and dog,* but not for man. Our work serves as another illustration of the point that the results with 1 species cannot always be applied to other species.

SUMMARY

1. The addition of cystine or methionine to a casein hydrolysate given as the sole source of nitrogenous food increased the rate of growth in rats.

2. When the casein hydrolysate was given by vein to dogs, the addition of methionine increased nitrogen retention, whereas the addition singly of the other 9 essential amino acids had no such effect.

3. In 4 groups of human subjects, the addition of methionine to a casein hydrolysate given as the sole source of nitrogenous food, did not increase nitrogen retention. This was true in surgical patients to whom the material was given intravenously, in infants fed a luxury level of nitrogen, and in well adults fed a maintenance nitrogen level.

4. When 4 human subjects were protein-depleted for 21 days, the subsequent administration of minimal amounts of an enzymic casein hydrolysate, with and without extra methionine, gave identical nitrogen retention.

5. These data are interpreted to mean that there is a species difference in the requirement for sulfur-containing amino acids, and that the human requirement for them is less than the requirement of the rat and dog, due presumably to a greater need for cystine in the building of hair.

6. The generally recognized nutritive difference between casein and lactalbumin is valid for the rat and for the dog, but not for man.

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THE CONJOINT EFFECTS OF VARIED INTAKES OF THIAMINE AND VITAMIN A ON RATS[1]

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This is a report of the fourth experiment in a series planned to show the effect of various dietary factors upon the utilization of vitamin A. In previous studies, it was found that increased caloric intake was responsible for greater weight gains than the unitage of vitamin A given, and that the percentage of fat and protein fed had no marked effect upon the utilization of vitamin A (Muelder and Kelly, '41, '42; Dye, Bateman and Porter, '45).

Few studies relating to the synergism of thiamine and vitamin A have been reported. In 1936, Randoin and Netter found that the young rat developed and matured rapidly in the presence of the B-vitamins whether or not vitamin A was present. It was not until the sixth week of their experimental low-vitamin A regime that the absence of vitamin A began to show its effect. Scheunert and co-workers ('37, '38) published papers on the effect of different quantities of vitamin A on the thiamine requirements and the effect of different quantities of thiamine on the vitamin A requirements of rats. Thiamine requirements of growing rats remained the same when the

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vitamin A intake varied from 5 to 2000 units daily. Furthermore, altering the thiamine intake did not alter the quantity of vitamin A required for curative purposes. More recently, Mitalo ('42) found that, if thiamine and vitamin A were both lacking in the diet, the loss of vitamin A from the livers of the experimental animals was more rapid than when vitamin A alone was absent from the diet. It is difficult to interpret these findings because of variations in experimental procedure. It seemed of interest to set up an experiment to test further the conjoint effects of varied intakes of thiamine and vitamin A.

EXPERIMENTAL PROCEDURE

Depletion period

At weaning, triads of rats of the same sex, weight, and litter were placed upon a vitamin-A-depletion diet of the following percentage composition: casein (A and thiamine extracted) 18, irradiated corn-starch 73, fat 5, and Osborne and Mendel salt mixture 4. Supplements of 0.5 mg autoclaved yeast and 3 μg of thiamine as thiamine chloride were administered daily. Water was given ad libitum. Weekly food consumptions and weight records were kept during this time. The criteria used for depletion were a plateau in weight and signs of xerophthalmia.

Experimental period

At depletion, rats in 2 series, one having the food intake controlled to the smallest quantity eaten by any member of the triad, and the other receiving the diet ad libitum were placed on various levels of vitamin A and thiamine. The levels of vitamin A were 0, $\frac{1}{2}$, $1\frac{1}{2}$, or 3 I.U. per day, combined at each level with 0, 6, or 9 μg of thiamine per day. When 4 rats of the same sex, weight and litter were available the fourth rat was fed 18 μg of thiamine per day. The experimental period lasted for 6 weeks during which daily food consumptions and weekly weight records were kept. At the end of the experimental period, the surviving animals were killed with chloro-

form and gross autopsy findings and length of the animal were recorded.

The average findings on food consumption, weight changes and length of rat were subjected to tests for significant differences according to the "t" formula.

RESULTS AND DISCUSSION

Male and female triads of rats were depleted of vitamin A in an average of 6.5 weeks. Average weekly food consumptions at the time of reaching a growth plateau ranged from 31 to 33 gm, with average weights ranging from 91 to 95 gm. The criteria used to evaluate the effect of varying levels of thiamine in the diet upon the utilization of vitamin A were (1) food intake, (2) weight change, (3) length of the rat at the end of the experimental period, and (4) number of foci of keratinized epithelial tissues, called "abscesses." Table 1 gives a summary of the results from the animals receiving a controlled food intake, and table 2, the results from animals receiving the diet ad libitum. Some results of feeding 6 I.U. of vitamin A were obtained. However, only the triads at levels of 0, 1½ and 3 I.U. of vitamin A will be considered in the discussion because of equalization of numbers and sex in these groups.

The daily addition of 6 or 9 μg of thiamine to the diet, whether or not vitamin A was present significantly increased food intakes and favorably affected weight changes in all groups of animals. For the controlled feeding group (table 1) members of the triad surviving beyond the death of the rat receiving no thiamine were permitted to eat 3 gm of food daily for the remainder of the 6-week experimental period. From the table it may be seen that these increased food intakes resulted in corresponding increased weight gains at all levels of vitamin A. The average weekly food consumption for all animals receiving no thiamine in the controlled feeding group was 11.3 gm. A similar weekly food consumption average, 11.2 gm, was observed for all animals having access to the diet ad libitum (table 2) when no thiamine was present, thus demonstrating the direct effect of thiamine upon appetite.

TABLE 1

Controlled feeding group: Average weights, food consumption, and autopsy findings on animals on a 6-week experimental period.

VITAMIN LEVELS		NUMBER OF RATS	WEEKLY AVERAGE			GAIN PER CALORIE INGESTED	LENGTH OF RAT AT END OF EXPERIMENT [1]	"ABSCESSES" EVIDENT AT AUTOPSY [2]
Thiamine	Vitamin A		Food intake [1]	Calorie intake	Weight change [1]			
μg	*I.U.*		*gm*		*gm*	*gm*	*in.*	*no.*
0	0	6	14.4 ± 1.5	59.0	— 9.9 ± 1.3	— 0.16	13.3 ± 0.0	3.2 ± 0.7
6	0	6	19.4 ± 0.8	79.5	— 3.4 ± 1.1	— 0.04	12.5 ± 0.2	6.0 ± 0.8
9	0	6	20.3 ± 0.6	83.2	— 2.7 ± 0.8	— 0.03	12.2 ± 0.5	4.7 ± 1.0
18	0	4	20.6 ± 0.5	84.5	— 3.2 ± 0.8	— 0.04	12.7 ± 0.0	6.8 ± 0.8
0	½	6	8.8 ± 0.9	36.1	— 13.7 ± 1.0	— 0.32	12.1 ± 1.4	2.3 ± 0.5
6	½	6	20.9 ± 0.1	85.7	— 0.3 ± 0.6	— 0.01	12.4 ± 0.4	1.5 ± 0.4
9	½	6	21.0 ± 0.1	86.1	— 0.4 ± 0.8	— 0.01	12.6 ± 0.2	1.3 ± 0.7
18	½	2	20.9 ± 0.1	85.7	+ 2.8 ± 0.8	+ 0.03	12.5 ± 0.0	1.5 ± 0.4
0	1½	6	11.4 ± 0.9	46.7	— 7.8 ± 1.5	— 0.17	12.1 ± 0.4	2.0 ± 0.6
6	1½	6	21.2 ± 0.4	86.9	+ 0.4 ± 0.9	+ 0.01	12.6 ± 0.3	0.3 ± 0.2
9	1½	6	21.3 ± 0.3	87.3	+ 1.4 ± 0.7	+ 0.02	12.4 ± 0.2	0.5 ± 0.3
18	1½	5	21.3 ± 0.4	87.3	+ 0.9 ± 0.6	+ 0.01	12.3 ± 0.1	0.6 ± 0.4
0	3	6	10.4 ± 1.0	42.6	— 11.6 ± 1.0	— 0.27	12.2 ± 0.3	1.7 ± 0.8
6	3	6	21.0 ± 0.7	86.1	+ 0.9 ± 0.7	+ 0.01	12.6 ± 0.0	0.7 ± 0.5
9	3	6	21.0 ± 0.9	86.1	+ 0.8 ± 0.7	+ 0.01	12.6 ± 0.1	0.2 ± 0.2
18	3	3	21.0 ± 0.0	86.1	+ 1.4 ± 0.9	+ 0.02	12.6 ± 0.3	0.7 ± 0.5

[1] Arithmetic means ± standard error.

[2] Areas that most frequently showed "abscesses" were middle ear, base of tongue, lymph glands in neck, salivary glands, and genito-urinary tract.

As would be expected, when the diet was available in quantities desired, the addition of 6 or 9 μg of thiamine to the diet stimulated increases in food intakes; and weight gains in corresponding groups were larger than those observed in the animals on controlled food intakes.

The absence of thiamine from the diet, with its resultant low food intake brought about marked losses in body weight (negative gain per calorie ingested) whether or not vitamin A was present or the animals were on controlled or ad libitum feeding. When both thiamine and vitamin A were present in the diet and the animals consuming their food ad libitum (table 2), 6 and 9 μg of thiamine produced identical gains in weight per calorie ingested. The slightly higher gain per calorie ingested (+ 0.04 gm) at the 3-unit level of vitamin A as compared to the response at either ½, or 1½ I.U. of vitamin A (+ 0.04 gm) may indicate a growth stimulating action of vitamin A. The gain per calorie ingested was lower on the limited calorie intake of the controlled feeding group (table 1) than for corresponding groups eating ad libitum. However, here again is some evidence of the growth promoting function of vitamin A in that 3 and 1½ I.U. of vitamin A produced a positive gain per calorie ingested whereas ½ I.U. of vitamin A produced a negative gain per calorie ingested on practically identical caloric intakes.

The daily addition of ½, 1½, or 3 I.U. vitamin A to the diet, in the absence of thiamine did not produce significant changes in food intakes or weight losses in the animal eating ad libitum as indicated by "t" tests and as shown in table 2. In groups of animals consuming controlled diets, however, the food intakes were somewhat lower and the weight losses greater (in the triads receiving ½ and 3 I.U. of vitamin A daily) than when neither vitamin A nor thiamine were present. In each case, 6 animals made up the group and it is likely that these numbers are too small for more than indications, but it is possible that an imbalance of vitamins with limited total caloric intake led to a poorer response to the food available.

TABLE 2

Ad libitum feeding group: Average weights, food consumption, and autopsy findings on animals on a 6-week experimental period.

VITAMIN LEVELS		NUMBER OF RATS	WEEKLY AVERAGE			GAIN PER CALORIE INGESTED	LENGTH OF RAT AT END OF EXPERIMENT [1]	"ABSCESSES" EVIDENT AT AUTOPSY [2]
Thiamine	Vitamin A		Food intake [1]	Calorie intake	Weight change [1]			
μg	*I.U.*		*gm*		*gm*	*gm*	*in.*	*no.*
0	0	6	11.5 ± 1.7	47.2	— 12.6 ± 1.2	— 0.27	11.8 ± 0.0	3.2 ± 0.8
6	0	6	26.9 ± 1.9	110.3	— 1.8 ± 2.0	— 0.16	12.9 ± 0.9	5.2 ± 0.7
9	0	6	23.9 ± 1.9	98.0	— 4.5 ± 1.9	— 0.46	12.4 ± 0.9	5.7 ± 0.7
18	0	5	29.9 ± 3.4	122.6	+ 0.1 ± 2.5	+ 0.01	13.9 ± 0.0	3.8 ± 1.0
0	½	4	10.9 ± 0.9	44.7	— 12.9 ± 1.5	— 0.29	12.0 ± 0.6	3.3 ± 0.9
6	½	4	33.8 ± 1.8	138.6	+ 5.0 ± 1.2	+ 0.04	12.5 ± 0.4	1.5 ± 0.8
9	½	4	33.6 ± 1.8	137.8	+ 5.1 ± 1.9	+ 0.04	12.3 ± 0.5	1.3 ± 0.3
18	½	1	23.2 ± 2.9	95.1	+ 2.3 ± 2.0	+ 0.02	11.5 ± 0.0	1.0 ± 0.0
0	1½	6	11.0 ± 1.2	45.1	— 11.6 ± 1.3	— 0.26	12.5 ± 0.0	1.3 ± 0.6
6	1½	6	44.3 ± 0.8	181.6	+ 8.1 ± 0.7	+ 0.04	13.6 ± 0.1	1.7 ± 0.4
9	1½	6	44.0 ± 1.4	180.4	+ 7.9 ± 1.3	+ 0.04	13.7 ± 0.2	1.5 ± 0.5
18	1½	3	39.7 ± 0.9	162.8	+ 6.2 ± 1.6	+ 0.04	13.3 ± 0.6	3.7 ± 0.7
0	3	3	11.4 ± 3.0	46.7	— 10.9 ± 1.7	— 0.23	12.9 ± 0.0	2.7 ± 0.9
6	3	3	41.3 ± 2.1	169.3	+ 7.9 ± 1.5	+ 0.05	13.4 ± 0.2	0.3 ± 0.3
9	3	3	47.3 ± 2.6	193.9	+ 9.5 ± 2.0	+ 0.05	13.8 ± 0.0	0.0 ± 0.0

[1] Arithmetic means ± standard error.

[2] Areas that most frequently showed "abscesses" were middle ear, base of tongue, lymph glands in neck, salivary glands, and genito-urinary tract.

When the relation of the response of the animals to the addition of vitamin A with known quantities of thiamine was tested statistically, vitamin A seemed to function as an essential factor in weight increases. In groups of animals fed ad libitum significant increases in food intake and decreases in weight loss or actual gains occurred with the addition of ½, 1½, and 3 I.U. of vitamin A with all corresponding levels of thiamine. In groups of animals with controlled food consumption, however, there were usually not significant increases in food intakes with the addition of vitamin A, but there were consistent and significant weight increases. Patterson, Henry and Crandall ('42) reported that rats which were pair-fed and received vitamin A were significantly heavier than control animals deprived only of that vitamin. The difference in weight was due to a greater retention of water, fat and protein in the animals supplied with the vitamin, and apparently was not due to alterations in absorption of food. In the present controlled-feeding groups, the difference between the average weekly weight changes in animals receiving 9 μg of thiamine and no vitamin A (— 2.7 gm) and those receiving 9 μg of thiamine and 3 I.U. vitamin A (+ 0.8 gm) was 3.5 gm. On the other hand, the difference between the average weekly weight gain of animals receiving 3 I.U. of vitamin A and no thiamine (— 11.6 gm) and those receiving 3 I.U. of vitamin A and 9 μg of thiamine (again + 0.8 gm) was 12.38 gm. A consideration of these differences in weight change, as ratios, seemed to indicate that both vitamins are essential for weight increases but that thiamine seemed to produce between 3 and 4 times the increase that vitamin A produced under the conditions of this experiment and in a 6-week period. It would be essential to know the composition of these weight changes before their relation to growth could be stated.

No significant effect on the length of the animal was observed with any of the different combinations of vitamin intake.

For all groups of animals, "abscesses" were highest in number when no vitamin A was present in the diet, regardless of

the presence of thiamine. In the controlled diet groups (table 1), "abscesses" averaged 3.2 per rat with no vitamin A or thiamine. The number of "abscesses" among animals eating ad libitum (table 2) averaged 3.2 per rat with no vitamin A or thiamine. When no vitamin A was fed, there were significant increases in the number of "abscesses" with the addition of thiamine in most groups of animals. In most instances, however, the addition of thiamine plus vitamin A was more effective in reducing the number of "abscesses" than was vitamin A alone. Since the presence or absence of "abscesses" is probably the most critical measure of vitamin A utilization applied in this study, the findings on the incidence of "abscesses" seems to indicate a synergistic effect of the 2 vitamins under discussion.

Thiamine independently and predominantly stimulated weight changes. Vitamin A, however, was also functioning as a weight-increase factor, at least in the presence of thiamine, but at what might be termed a "slower rate." The reasons for the slightly lower food intakes and greater weight losses among animals fed iso-caloric diets with vitamin A and no thiamine are not clear from the criteria used in this study, but are probably not significant. That there were synergistic effects of thiamine on the utilization of vitamin A in vitamin A depleted rats were not strikingly demonstrated by these data, although they seem to be indicated in the findings on the incidence of "abscesses."

SUMMARY

The effect of thiamine on the utilization of vitamin A was studied by means of food intake, weight change, length of rat, and number of foci of keratinized epithelial tissues called "abscesses" in animals depleted of vitamin A.

The daily addition of 6, or 9 μg of thiamine to the diet, whether or not vitamin A was present, significantly increased food intakes and favorably affected weight changes in all groups of animals. On the other hand, the daily addition of ½, 1½, or 3 I.U. of vitamin A, in the absence of thiamine, did

not produce significant changes in food intakes or weight losses in animals eating ad libitum of a basal vitamin A and thiamine free diet. In groups of animals on iso-caloric intakes, however, food intakes were somewhat lower and weight losses greater when ½ or 3 I.U. of vitamin A were given, than when neither vitamin A nor thiamine were present in the food. Vitamin A functioned as an essential factor in weight increases in the presence of thiamine.

No significant differences in the length of the rats were observed among the groups, regardless of vitamin intake in the 6-week period.

For all groups of animals, "abscesses" were highest in number when no vitamin A was present in the diet. When no vitamin A and some thiamine were fed, there were significant increases in the number of "abscesses" in several groups of animals. In most instances, however, the addition of thiamine plus vitamin A was more effective in reducing the number of "abscesses" than was vitamin A alone.

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THE VALUE OF MEAT AND PEAS, ALONE OR IN COMBINATION, AS A SOURCE OF PROTEIN FOR GROWTH[1]

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The proteins of meat are considered of high nutritive quality. Hoagland and Snider ('26, '27) reported them to be adequate for growth in rats when they supplied protein at a 10% level and to be very efficient supplements for the cereals and for white flour. Clayton ('30), also working with rats, obtained growth, maintenance and reproduction with beef protein at a 20% level. McCollum et al. ('21) found meats valuable supplements for cereal seeds but not so satisfactory as supplements for legume seeds.

Woods et al. ('43) reported that the slow growth in rats on the protein of raw Alaska peas could be overcome by the addition of methionine. Bolin and associates ('46) found that for chick growth, various animal and plant protein supplements to pea meal rations were greatly improved by the addition of methionine. Recent investigations at this station by Beeson and Hickman ('45, '45a) and Lehrer et al. ('46, '46a) have shown that peas, when supplemented with a small quantity of meat meal or good pasture, are an excellent source of protein when fattening swine.

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In low-cost diets or when meats are difficult to obtain, a very common dietary recommendation is an increased use of legumes to meet the protein requirement. The study reported here was designed to compare, at a critical level, the proteins of pork, lamb, beef and peas and to determine the effect upon growth of replacing a part of the meat of the diet with a serving of peas.

EXPERIMENTAL PROCEDURE

Weanling rats housed in individual cages and weighed weekly for 8 weeks were used as test animals. Food consumption records were made at each weighing.

Alaska field peas, grade no. 1, were soaked, cooked and dried without discarding any of the liquid and then finely ground.

The meats were prepared and roasted as recommended by the National Livestock and Meat Board ('45). The internal temperatures of the various cuts were as follows: for fresh ham, 185°F.; leg of lamb, 180°F.; and beef round, 170°F. All bone and all visible fat were removed from the meats before they were ground. They were dehydrated at a temperature of 144°F. in the same manner as the peas and were stored under refrigeration. A diet in which dried whole egg furnished the protein was used in these experiments as a standard of comparison. Animals on the egg diet exhibited good growth with efficient utilization of protein.

The dried cooked peas and the dried meats were analyzed for nitrogen and fat. The diets were made up to contain 10% protein ($N \times 6.25$) and were adjusted to the same fat level by the use of refined cottonseed oil. The composition of the diets is given in table 1.

For compounding the diets in the study of peas as an extender of meats, 50 gm of peas and 4 oz. of fresh meat were taken as average servings of these foods in human dietaries. In those diets marked "a" the protein was derived from a mixture of peas and meat in the relationship of 1 serving of peas (50 gm) to ¼ serving of fresh meat (1 oz.). In those

TABLE 1

Composition of diets.[1]
(Source of protein calculated to 10% protein level.)

LOT NO.	PROTEIN SOURCE		SUGAR	SALT MIXTURE	COTTON-SEED OIL	COD LIVER OIL	AGAR-AGAR
		gm/kg	*gm/kg*	*gm/kg*	*gm/kg*	*gm/kg*	*gm/kg*
1	Egg	227	639.5	40	30	20	20
2	Pork	154	738	40	4.5	20	20
3	Lamb	135	729.5	40	32	20	20
4	Beef	142	723	40	31.5	20	20
5	Peas	433	444.5	40	39	20	..
6	Peas	433					
	and		441.5	40	39	20	..
	dl methionine	3					
7	Peas	320					
	and		526.5	40	30	20	..
	Pork (a)	40					
8	Peas	254					
	and		575	40	24.5	20	..
	Pork (b)	63					
9	Peas	310					
	and		530.5	40	37	20	..
	Lamb (a)	39					
10	Peas	241					
	and		580	40	35.5	20	..
	Lamb (b)	60					
11	Peas	314					
	and		526.5	40	37	20	..
	Beef (a)	39					
12	Peas	246					
	and		574	40	35.5	20	..
	Beef (b)	61					

[1] Each diet contained 21 gm/kg of vitamin-sugar mixture in addition to 2.5 gm/kg of choline chloride.

(a) Mixed in proportion of ¼ serving (1 oz.) of *fresh* meat to 1 serving (50 gm) of peas.

(b) Mixed in proportion of ½ serving (2 oz.) of *fresh* meat to 1 serving (50 gm) of peas.

marked "b" the relationship was that of 1 serving of peas to ½ serving (2 oz.) of fresh meat. The protein of these mixtures was fed at the 10% level as in the other diets.

Pure vitamins of the B-complex were mixed with sugar, and 21 gm of the mixture included in each kilo, providing the following supplements per 100 gm of diet: thiamine, 0.49 mg; riboflavin, 0.49 mg; pyridoxine, 0.61 mg; nicotinic acid, 0.61 mg; calcium pantothenate, 4.9 mg; para-amino benzoic acid, 29.4 mg; and inositol, 98.0 mg. The Osborne and Mendel ('19) salt mixture was used in all diets.

Statistical analyses of the data were made according to the methods of Snedecor ('40).

RESULTS AND DISCUSSION

The results of this series of experiments are summarized in tables 2 and 3.

On the basis of daily gains in weight the pork, lamb, and beef compared favorably with egg, but the peas were significantly inferior (table 3). From the standpoint of efficiency as measured by gain per gm of protein consumed, pork, lamb and beef were not significantly different from egg, but the peas were markedly less efficient.

When cooked peas were supplemented with 0.3% dl-methionine the average daily gain was tripled and the protein efficiency was more than doubled, confirming earlier published findings of Woods and co-workers ('43) that peas lack methionine for normal growth. Although the peas and methionine diet was not as efficient as the whole egg diet, the daily gains were not significantly different.

The rats on the pea diet, in addition to being smaller, had short, soft hair and their average survival period was only 33 days. A postmortem [3] examination revealed a characteristically abnormal liver. The liver condition was typical of acute hepatitis and characterized by 2 progressive degrees of involvement — cloudy swelling and fatty degeneration.

[3] Credit is due Dr. G. C. Holm, Station Veterinarian, for valuable assistance in postmortem studies.

The stage of liver damage was dependent upon the length of time the rat showed retarded growth. No cases of cirrhosis of the liver were observed, which can be attributed to the acute nature of the involvement. Occasionally hemorrhage in the liver was encountered. These conditions did not occur in rats receiving the pea diet supplemented with adequate methionine.

In diets in which the peas and meats were combined, at both levels of meat, the average daily gain and the efficiency of the

TABLE 2

Average growth response.

LOT NO.	DIET	NO. OF RATS	AV. DAILY GAIN	AV. GAIN PER GM OF PROTEIN	AV. FOOD REQUIRED PER GM GAIN	AV. FOOD CONSUMED DAILY	AV. LIFE OF RATS [1]
			gm	*gm*	*gm*	*gm*	*days*
1	Egg	8	2.17 ± .11	2.50 ± .08	3.99 ± .20	8.65 ± .36	56
2	Pork	9	2.00 ± .11	2.16 ± .10	4.63 ± .24	9.28 ± .26	56
3	Lamb	9	2.34 ± .12	2.32 ± .11	4.31 ± .26	10.09 ± .31	56
4	Beef	9	2.35 ± .09	2.19 ± .05	4.56 ± .11	10.75 ± .33	56
5	Peas	19	0.60 ± .04	0.95 ± .06	10.54 ± .68	6.36 ± .45	33
6	Peas and 0.3% dl-methionine	19	1.88 ± .09	2.04 ± .07	4.89 ± .13	9.20 ± .39	56
7	Peas and Pork (a)	9	1.38 ± .17	1.94 ± .24	5.16 ± .66	7.15 ± .43	21
8	Peas and Pork (b)	9	1.62 ± .10	1.70 ± .10	5.88 ± .30	9.54 ± .59	42
9	Peas and Lamb (a)	9	1.66 ± .13	2.41 ± .07	4.15 ± .13	6.89 ± .43	21
10	Peas and Lamb (b)	9	1.80 ± .08	1.89 ± .14	5.30 ± .36	9.51 ± .56	41
11	Peas and Beef (a)	9	1.38 ± .19	1.96 ± .17	5.10 ± .70	7.03 ± .54	25
12	Peas and Beef (b)	9	1.85 ± .03	1.95 ± .15	5.42 ± .33	10.00 ± .55	48

[1] Experimental period 56 days.

TABLE 3

Statistical comparisons of growth on various diets.

LOT NO.	DIETS COMPARED	AVERAGE DAILY GAIN (GM)			AVERAGE GAIN PER GRAM PROTEIN (GM)		
		Mean difference	Standard error of difference	t [1]	Mean difference	Standard error of difference	t [1]
1–2	Egg vs. Pork	0.17	.16	1.06	0.34	.13	2.66
1–3	Egg vs. Lamb	0.17	.16	1.06	0.18	.14	1.28
1–4	Egg vs. Beef	0.18	.14	1.28	0.31	.09	3.44
1–5	Egg vs. Peas	1.57	.12	13.08	1.55	.10	15.50
2–3	Pork vs. Lamb	0.34	.16	2.13	0.16	.15	1.07
2–4	Pork vs. Beef	0.35	.14	2.50	0.03	.11	0.03
7–9	Peas + Pork (a) vs. Peas + Lamb (a)	0.28	.22	1.27	0.47	.25	1.88
7–11	Peas + Pork (a) vs. Peas + Beef (a)	0.00	.25	0.00	0.02	.29	0.06
8–10	Peas + Pork (b) vs. Peas + Lamb (b)	0.18	.13	1.38	0.19	.17	1.12
8–12	Peas + Pork (b) vs. Peas + Beef (b)	0.23	.10	2.30	0.25	.18	1.39
5–6	Peas vs. Peas + 0.3% dl-methionine	1.28	.10	12.80	1.09	.09	12.11
1–6	Egg vs. Peas + 0.3% dl-methionine	0.29	.14	2.07	0.46	.11	4.18
6–7	Peas + 0.3% dl-methionine vs. Peas + Pork (a)	0.50	.19	2.63	0.10	.25	0.40
6–8	Peas + 0.3% dl-methionine vs. Peas + Pork (b)	0.26	.13	2.00	0.34	.12	2.83
6–9	Peas + 0.3% dl-methionine vs. Peas + Lamb (a)	0.22	.16	1.38	0.37	.10	3.70
6–10	Peas + 0.3% dl-methionine vs. Peas + Lamb (b)	0.08	.12	0.67	0.15	.16	0.94
6–11	Peas + 0.3% dl-methionine vs. Peas + Beef (a)	0.50	.21	2.38	0.08	.18	0.44
6–12	Peas + 0.3% dl-methionine vs. Peas + Beef (b)	0.03	.09	0.33	0.09	.17	0.53

[1] A t value of 3.499 establishes the 1% point for the comparisons based on the smallest number of degrees of freedom; therefore a value of 3.499 or larger is highly significant.

proteins were significantly increased over diets in which peas alone supplied the protein. The peas and meat combination diets gave average daily gains that were not significantly different from those obtained with the peas and methionine diet but on none of the pea and meat combination diets did all the animals live the entire test period. On the lower level of meat the average survival time was less than ½ the test period and on the higher level 6 to 7 weeks. Rats on these diets made good growth but died suddenly without previous indications of disorder. On autopsy they revealed a liver condition of varying severity similar to the one found in the rats on peas.

Since this liver condition was prevented by adequate methionine supplements to the pea diet it appears that the meats in the amounts here used did not furnish sufficient methionine to provide for the increased growth and for maintenance of normal liver tissue.

If the methionine content of voluntary muscle protein is estimated at 3%, which is approximately the value reported by Beach et al. ('43) from chemical analyses of beef, pork and lamb muscle, the amounts of methionine added to each kilo of diet by the meat supplements actually used in these experiments were about 0.8 gm by the smaller and 1.3 gm by the larger supplements. These values are considerably below the 3 gm per kilo added in the 0.3% dl-methionine supplemented pea diet. Mitchell and Block ('46) in their comparison of analytical data for different food proteins with those of dried whole egg note that for beef muscle the indicated limiting amino acid is the methionine plus cystine combination. These figures suggest that the peas and meat combination diets at both levels of supplementary meat were limited by their content of methionine but do not explain the acceleration of growth at the expense of liver tissues.

SUMMARY

Cooked pork, lamb, beef, and peas, and some combinations of peas and the meats, have been compared with whole egg as sources of protein for growth in young rats.

Measured by growth rate and by efficiency the various meats, when fed at a 10% protein level, were as good sources of protein for growth as were eggs.

Cooked peas alone were a poor source of protein for growth whether measured by average daily gain or by gain per gram of protein. When peas were supplemented with 0.3% dl-methionine, growth was increased until it was not significantly different from that obtained with whole egg protein although it was not as efficient.

At the levels of meat fed in combination with peas in this investigation growth was improved over that from peas alone but even the meat and pea diets were inadequate as evidenced by a characteristic liver involvement and early death of many of the test animals.

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TISSUE RESERVES OF ASCORBIC ACID IN NORMAL ADULTS ON THREE LEVELS OF INTAKE [1]

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ONE FIGURE

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Nutrition workers are not yet agreed on a desirable nutritive state with respect to vitamin C, nor on the intake of ascorbic acid which should be recommended to maintain such a state. The recommended allowance of the National Research Council, 70 mg for women and 75 mg for men, is insufficient to maintain tissue saturation in adults but provides a wide margin of safety over the amount required for prevention of scurvy. The League of Nations standard allowance of 30 mg daily has been commonly accepted in Europe, and is recognized in the United States as the minimum requirement to be stated in food labeling.

The present study is concerned with a comparison of tissue stores of ascorbic acid associated with 3 levels of intake which had been maintained for a period of 6 weeks. The intakes used were 70 mg, 50 or 53 mg, and 33 mg, respectively. The

[1] Some of the data in this paper are taken from theses presented to the faculty of the Graduate School of Cornell University in fulfillment of the requirement for the M.S. degree, by Ann C. Moore, June 1944, Margaret A. Delaney, October 1945, and J. Estelle Haines, February 1946. Further details will be found in the theses.

highest level of intake studied approximated the National Research Council allowance, the lowest approximated the League of Nations standard, whereas in the other 2 experiments, 1 with natural food and the other with a synthetic diet, the intakes were intermediate.

The state of the subjects' tissue reserves of ascorbic acid at the end of the 6-week periods was judged by the fasting plasma ascorbic acid values and whether or not these values had reached a plateau, as well as by the response to standard test doses, and the number of doses given daily which were required to restore tissue saturation.

The experimental period selected, 6 weeks, is one in which the fasting plasma ascorbic acid value reached zero for a subject whose diet was devoid of ascorbic acid (Crandon, Lund and Dill, '40), hence it was believed to be long enough to permit the demonstration of differences in body stores of ascorbic acid on these levels of intake, should such differences exist.

EXPERIMENTAL

Subjects

Twelve normal adults, 6 men and 6 women, ranging in age from just under 20 to 46 years, served as subjects. Four of these served for 2 experimental periods each. Data concerning the sex, age, height and weight of the subjects are presented in table 1. Before each experiment, the subjects were examined by a physician at the Student Medical Clinic of Cornell University and were found to be in good physical condition.[2] Six of the subjects had mild infections of the upper respiratory tract,[3] without fever, in the course of the experiments which were conducted during the winter. Subject J. M. had a cold with slight fever (99.6°F.) for 3 days during the sixth week of the experiment.

[2] Subjects E.K. and H.G. had poliomyelitis in childhood; subject J.M. had asthma in 1931, with little difficulty since.

[3] Although the subjects had been instructed not to take any medication during the experimental period, 1 subject took 110 grains of aspirin in a 32-hour period in the fifth week of the experiment, without apparent effect on the urinary excretion or fasting plasma values of ascorbic acid.

TABLE 1

Sex, age, height and weight of 12 subjects[1] on controlled intakes of ascorbic acid for 6-week periods.

SUBJECT	AGE	HEIGHT		WEIGHT[2]		SUBJECT	AGE	HEIGHT		WEIGHT[2]	
	yr.	*inches*	*cm*	*lb.*	*kg*		*yr.*	*inches*	*cm*	*lb.*	*kg*
	Intake: 33 mg/day						*Intake: 53 mg/day*				
EH ♀	22	66½	169	134	61	AM ♀	23	67½	172	136	62
SP ♂	19	67	170	140	64	DL ♀	30	66	168	143	65
HG ♂	26	72½	184	127	58	EK ♂	24	69	175	154	70
LG ♀	29	67	170	153	69	HG ♂	26	72½	184	133	60
	Intake: 50 mg/day						*Intake: 70 mg/day*				
JES ♀	21	63	160	128	58	AM ♀	22	67½	172	134	61
DL ♀	31	66	168	133	60	PH[3] ♂	31	68½	174	141	64
MH ♀	46	67	170	138	63	EK ♂	23	69	175	163	74
						JM ♂	24	68	173	163	74
						JL ♂	23	67	170	152	69

[1] Subjects HG, DL, AM and EK served for 2 experimental periods.

[2] Average of daily weights during 6-week period. Nude weight obtained by subtracting the weight of the usual amount of clothing from the daily weight.

[3] Subject PH is Chinese.

Diet and ascorbic acid intake

The basal diet for 3 of the 4 experimental periods was similar to that reported by Lewis et al. ('43). This diet provided from 10 to 13 mg of ascorbic acid, and was supplemented by doses of 20, 40 and 60 mg of synthetic ascorbic acid,[4] taken after breakfast. These doses, together with the ascorbic acid content of the basal diet, brought the total ascorbic acid intake to 33, 53 and 70 mg, respectively, in the 3 experiments. In the study on the 53-mg intake, the diet was modified so as to keep the thiamine intake constant for each subject (Gifft and Hauck, '46). In the fourth experiment, which was designed primarily to study thiamine metabolism (Hathaway and Strom, '46) a synthetic diet was used, including 50 mg of ascorbic acid.

[4] Acknowledgment is made to Hoffmann-LaRoche, Inc., for a generous supply of synthetic ascorbic acid.

Plan of the experiment

Preliminary saturation period. In order to assure full tissue stores for all subjects at the beginning of the experiments, they were given either the juice of 8 oranges, or 400 mg ascorbic acid daily for 3 days in addition to their usual diets. On the fourth day, subjects were given either the basal diet, or other food selected for its low ascorbic acid content, plus a test dose of 400 mg of ascorbic acid, 200 mg with breakfast and 200 mg with luncheon. Urinary excretions of ascorbic acid on this day were from 250 to 387 mg, well in excess of 50% of the test dose. Fasting plasma ascorbic acid values were 1.2 mg % or more for all subjects at this time.

Experimental period. The preliminary saturation period was followed by a period of 6 weeks on the level of intake under investigation, i.e., 33, 50, 53 and 70 mg per day, respectively, in the 4 experiments.

Resaturation period. Following the period of 6 weeks on a constant level of intake, subjects were given daily 400 mg test doses of ascorbic acid, 200 mg with breakfast and 200 mg with luncheon, until the 24-hour urinary excretion of ascorbic acid exceeded 50% of the test dose. The subjects were then considered to be saturated with respect to ascorbic acid.

Ascorbic acid determinations made. Daily determinations were made of the ascorbic acid content of the fasting plasma and 24-hour urine samples. Each 24-hour period ended with the collection of the specimen voided ½ hour before the fasting blood sample was taken. The fasting plasma level was considered to be associated with the previous day's intake; therefore, in making calculations, the fasting plasma values were paired with the previous day's urinary excretion values.

In order to estimate the subjects' renal thresholds for ascorbic acid, determinations were made of the hourly excretion of ascorbic acid, both at fasting and following ingestion of ascorbic acid, together with the corresponding plasma ascorbic acid values. Some of these data are presented here, since they provide additional evidence concerning the state of the

subjects' tissue stores at the beginning and end of the experiment. The estimation of the renal threshold is reported in a separate publication (Klosterman et al., '47).

Analytical methods

Each urine specimen, or an aliquot portion thereof, was acidified immediately with 10% by volume of 2 N sulfuric acid containing 2% of metaphosphoric acid. The acidified 24-hour specimens, or appropriate aliquot portions thereof, were refrigerated and pooled for analysis. The ascorbic acid content of the urine was determined by titration with a buffered solution of the indicator, sodium 2, 6-dichlorobenzenone-indophenol,[5] standardized twice weekly against a known solution of ascorbic acid.

Reduced ascorbic acid in plasma was determined by the micromethod of Farmer and Abt ('36).

The ascorbic acid content of foods in the basal diet was determined 3 or more times during each experimental period, using the indophenol titration method of Bessey ('38a) modified by the use of the Waring Blendor (Davis, '39).

RESULTS AND DISCUSSION

The results of these experiments are summarized in tables 2 and 3.

Evidence on the tissue reserves, obtained from the 24-hour excretion of ascorbic acid, was corroborated by the hourly determinations of urinary excretion and plasma levels of ascorbic acid, made in connection with the estimation of the subjects' renal thresholds. Typical curves obtained from such studies are presented in figure 1, A and B. Curves marked 1 are representative of values obtained on the first day of the 6-week period. The fasting plasma values for all subjects were near their renal thresholds for ascorbic acid, and were maintained above the threshold value for several hours by the small daily supplement. This behavior is typical of subjects

[5] Eastman Kodak Company.

TABLE 2

Fasting plasma ascorbic acid values for adult subjects on various levels of intake during a 6-week period, and in response to 400-mg test doses at the end of the period.

INTAKE AND SUBJECT	MEAN FASTING PLASMA ASCORBIC ACID VALUES IN MG %						RESPONSE TO 400 MG TEST DOSES, FASTING PLASMA ASCORBIC ACID VALUES, MG %					R.T.[1]
	Week[2]						Day					
	1	2	3	4	5	6	1	2	3	4	5	
70 mg												
A.M.♀	1.07	1.00	0.89	0.86	0.89	0.86	1.5	1.3				1.2
P.H.♂	0.76	0.90	0.66	0.67	0.70	0.67	1.1	1.1				1.05
E.K.♂	0.89	0.76	0.64	0.61	0.57	0.50	0.5	1.0	1.3	1.2		1.1
J.M.♂	1.06	0.94	0.70	0.63	0.63	0.63[3](6)	0.9	1.2	1.1			1.1
J.L.♂	0.83	0.76	0.61	0.67	0.66	0.66	1.1	1.2				1.0
53 mg												
A.M.♀	1.22	0.83	0.77	0.74	0.63	0.63	1.0	1.3	1.5			1.2
E.K.♂	1.03	0.67	0.57	0.48	0.44	0.42	0.6	0.9	1.2	1.2	1.4	1.1
H.G.♂	1.04	0.71	0.61	0.53	0.48	0.46	1.1	1.1	1.3	1.4		1.1
D.L.♀	1.25	0.85	0.78	0.69	0.61	0.53	1.1	1.4	1.6			1.15
50 mg[4]												
D.L.♀	1.16(5)	0.98(5)	0.94(5)	0.81	0.84	0.59	1.0	1.3	1.3			1.15
J.E.S.♀	1.15(6)	1.03(6)	1.02(4)	0.83(6)	0.77	0.59	1.1	1.4	1.4			1.3
M.H.♀	0.93(6)	0.73(6)	0.73(3)	0.63	0.60	0.50	0.8	1.0	0.9	1.1	1.0[6]	1.0
33 mg												
E.H.♀	1.00	0.69	0.54	0.40	0.37	0.34	0.6	0.9	1.5	1.3		1.3
S.P.♂	0.96	0.60	0.50	0.41	0.34	0.36	0.7	1.2	1.3	1.5		1.3
H.G.♂	0.93	0.63	0.51	0.41	0.37	0.28(6)	0.6	0.7	1.2	1.4	1.5	1.1
L.G.♀	0.93	0.61	0.49	0.41	0.34	0.31	0.6	0.8	1.4	[5]	1.6	1.3

[1] Renal threshold. Data from Klosterman et al. ('47).

[2] Weekly means were calculated from 7 daily values except where a figure in parentheses is given to indicate that the mean was derived from fewer values.

[3] This subject had a cold with fever for 3 days during the sixth week of the experiment.

[4] Synthetic diet used. In all other studies the basal diet was composed of foods low in ascorbic acid content.

[5] This subject had a mild illness without fever on the fourth day of the resaturation period. Since no fasting plasma determination was made on this day, the experiment was continued 1 day beyond the time when more than 50% of the test dose was excreted in 24 hours.

[6] Fasting plasma ascorbic acid values for this subject were at or above her renal threshold for the next 2 days.

whose tissue reserves of ascorbic acid are full. Similar plasma ascorbic acid curves for A.M. and J.L. on the second morning of the resaturation period indicated that their tissue stores had been refilled. This was borne out by the excretion of over

TABLE 3

Twenty-four hour urinary ascorbic acid values for adult subjects on various levels of intake during a 6-week period, and in response to 400-mg test doses at the end of the period.

INTAKE AND SUBJECT	MEAN 24-HOUR URINARY EXCRETION VALUES FOR ASCORBIC ACID IN MG						RESPONSE TO 400 MG TEST DOSES, 24-HOUR URINARY EXCRETION VALUES FOR ASCORBIC ACID IN MG						
	Week [1]						Day						
	1	2	3	4	5	6	1	2	3	4	5	6	7
70 mg													
A.M.♀	49	21	21	24	24	23	115	266					
P.H.♂	53	20	21	22	26	25	67	253					
E.K.♂	43	33	32	30	30	32	36	67	199	294			
J.M.♂	46	19	20	23	26	28 [2]	32	190	334				
J.L.♂	41	21	22	25	23	23	125	236					
53 mg													
A.M.♀	40	19	19	19	19	17	28	155	252				
E.K.♂	30	24	25	25(6)	24	22	24	25	39	152	242		
H.G.♂	42	21	22	20	19	17	18	84	182	302			
D.L.♀	51	23	22	21(6)	22	17	27	62	215				
50 mg [3]													
D.L.♀	34(6)	22	17(6)	17	15	15	25	110	222				
J.E.S.♀	34	23	20(6)	21	19	19	24	85	222				
M.H.♀	34	22	16	17	15	14	15	32	61	117	124	148	183
33 mg													
E.H.♀	37(6)	26	27(6)	27	24	22	25	28	129	263			
S.P.♂	28	20	23	21	19	17	24	29	108	263			
H.G.♂	37	23	22(6)	21	17	15	25	23	30	175	212		
L.G.♀	30	19	21	19	18	16	20	21	55	230	295 [4]		

[1] Weekly means were calculated from 7 daily values except where a figure in parentheses is given to indicate that the mean was derived from fewer values.

[2] This subject had a cold with fever for 3 days during the sixth week of the experiment.

[3] Synthetic diet used. In all other studies the basal diet was composed of foods low in ascorbic acid content.

[4] This subject had a mild illness without fever on the fourth day of the resaturation period. Since no fasting plasma determination was made on this day, the experiment was continued 1 day beyond the time when more than 50% of the test dose was excreted in 24 hours.

50% of the test dose by these subjects on this day. Note, however, that the low fasting plasma values for subjects E.H. and H.G. following the period on a 33-mg intake, were raised only slightly by the 200 mg test dose on the first day of the resaturation period. On the third day, the test dose caused the plasma level of ascorbic acid to exceed the renal threshold for a short time, but the tissues were still avidly taking up ascorbic acid, so that little urinary excretion occurred. As judged by the 24-hour urinary excretion in response to the test dose E.H. required 1 more, and H.G. 2 more days to become saturated.

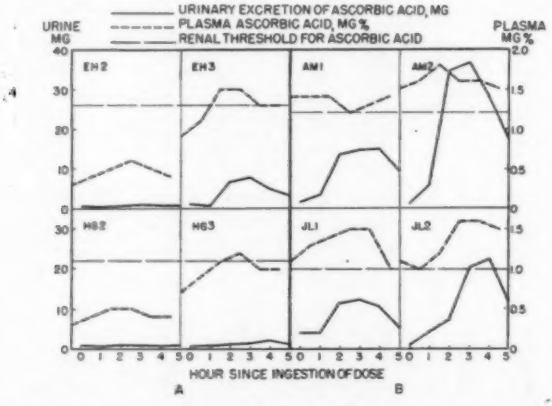

Fig. 1 Plasma ascorbic acid and urinary excretion curves following ingestion of ascorbic acid. In each case the dose of ascorbic acid was taken at the hour marked 0, immediately following the collection of a 1-hour fasting specimen of urine, and ½ hour after the fasting blood sample was taken. Each subject's renal threshold for ascorbic acid is indicated by a line parallel to the base line. Fig. 1 A and B include representative curves for subjects on the 33 mg and 70 mg intakes, respectively.

Curves marked 1 represent data obtained on the first day of the experiment, following saturation when A.M. and J.L. received 60 mg doses of ascorbic acid at the hour marked 0.

Curves marked 2 and 3 represent data obtained during the resaturation period, when a 200 mg test dose was given at the hour marked 0. Curves marked E.H. 2, H.G. 2 and E.H. 3, H.G. 3 represent studies made on the first and third mornings of the resaturation period; curves marked A.M. 2 and J.L. 2 represent data obtained on the second morning of the resaturation period.

In comparing the results of experiments on the 3 levels of intake, the consistent behavior of subjects who served for 2 experiments is noteworthy. Thus A.M. required 2 days for resaturation on the 70 mg intake, and 3 days on the 53 mg intake; E.K. required 4 days on the 70 mg intake and 5 days on the 53 mg intake; H.G. required 4 days on the 53 mg intake and 5 days on the 33 mg intake; and D.L. required 3 days for resaturation after periods on 53 and 50 mg intakes. There was no indication that D.L.'s ascorbic acid metabolism on the synthetic diet differed from her metabolism when the basal diet was composed of ordinary foods.

In general, the reducing value of E.K.'s 24-hour specimens of urine was higher than for the other subjects, after the first week of the study. This may have been related to a higher excretion of non-ascorbic acid reducing substances by this subject, since in a series of determinations with the photoelectric colorimeter by the method of Bessey ('38b), the mean urinary ascorbic acid excretion was 14 mg less than when estimated by direct titration, as compared with the following differences for other subjects studied at the same time: P.H., 6; A.M., 7; J.M. and J.L., 9 mg. We have found no constant relationship between the ascorbic acid values as estimated by these 2 methods.

No explanation is apparent for the anomalous behavior of subject M.H. who did not excrete over 50% of the test dose even after 7 days, although her fasting plasma ascorbic acid value reached her renal threshold on the second day, and hovered about this concentration until the experiment was terminated because the subject left town. She was capable of excreting over 50% of the test dose because she did so during the preliminary saturation period.

Although the 70 mg intake of ascorbic acid was not sufficient to maintain any of the 5 subjects in a state of tissue saturation, 4 of the 5 reached a plateau in urinary excretion of ascorbic acid after the first week and in fasting plasma ascorbic acid values after the second week on this intake (tables 2 and 3). Probably these subjects had reached a stable

state in which the tissue reserves were being maintained at a level slightly below saturation. The prompt response of both plasma and urinary excretion values during the resaturation period indicates that except for subject E.K., these adults had suffered only slight depletion of their tissue stores of ascorbic acid in 6 weeks on the 70 mg intake. In a large scale experiment, Linghorne et al. ('46) found that slightly higher intakes, approximately 75 and 80 mg per day, maintained plasma values between 0.7 and 1.0 mg % in young R.C.A.F. personnel for a period of 8 months.

In contrast to the relatively good stores on the intake which approximated the National Research Council allowance of ascorbic acid, marked depletion of the tissue stores occurred in 6 weeks on the 33 mg intake, which approximates the League of Nations standard. This depletion may not have reached its maximum for this intake, for the fasting plasma ascorbic acid and urinary excretion values had not reached a definite plateau in the 6 weeks (tables 2 and 3). Possibly the values would not have fallen much lower had the experiment been continued, for Linghorne et al. ('46) observed only slightly lower plasma values, approximately 0.25 mg % for subjects who were maintained on a 25 mg intake for 8 months.

As would be expected, the results on the intermediate intakes were intermediate between those on the intakes approximating the National Research Council allowance and the League of Nations standard. These experiments provide no evidence concerning the value of tissue reserves of ascorbic acid from the standpoint of health.

SUMMARY

The ascorbic acid metabolism of normal adults was studied on 3 levels of intake for periods of 6 weeks, followed by resaturation periods. Five subjects received the 70 mg intake, which approximated the National Research Council allowance; 6 subjects received either an intake of 50 mg of ascorbic acid as part of a synthetic diet, or 53 mg, with a basal diet of ordinary foods, 1 subject serving for both experiments; 4

subjects received an intake of 33 mg, which approximated the League of Nations standard allowance. Depletion of tissue stores was slight on the 70 mg allowance, moderate on the allowance of 50 to 53 mg, and fairly marked on the 33 mg intake, without evidence that the maximum depletion had been reached at the end of the 6-week period on this intake.

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